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RESEMBLANCES BETWEEN THE ELECTROMOTOR VARIATIONS OF RHYTHMICALLY REACTING LIVING AND NON-LIVING SYSTEMS

By RALPH S. LILLIE

(From the Laboratory of General Physiology, University of Chicago, Chicago)

PLATES 1 AND 2

(Accepted for publication, May 3, 1929)

It is well known that under certain conditions the reaction of iron wire with nitric acid exhibits an automatic rhythm of sometimes remarkable regularity, consisting in an alternation of active and passive periods.[1] In a recent study of this phenomenon[2] I have found the most regular rhythms in soft iron wire of low carbon content. Wire drawn from iron prepared by electrolysis of pure solutions of iron salts[3] and the pure commercial soft iron known as Armco[4] are especially favorable. In hard steel wire (piano wire) the tendency to rhythm is slight, and any rhythm shown is local and irregular. In general the essential conditions for a regular and rapid rhythm (*e.g.*, 50 to 100 per minute), involving all parts of a wire of some length (10 cm. and more), may be defined as follows: (1) The acid must be of sufficient strength (> 55 v.%), such that the passive state is one of stable equilibrium; the wire then reverts automatically to the passive state after each activation (spontaneous repassivation); (2) the wire must transmit states of activation readily and quickly; (3) it must recover its transmissivity rapidly after activation; and (4)

[1] For a comprehensive account of rhythmical reactions in metals and in inorganic systems in general *cf.* the monograph by Kremann, R., "Die periodischen Erscheinungen in der Chemie," *Ahrens Sammlung chemischer und chemisch-technischer Vorträge*, 1913, **19**, p. 289; also the recent book by Hedges and Myers, "The Problem of Physico-chemical Periodicity," London, E. Arnold & Co., 1926.

[2] Lillie, R. S., *Archivio di Scienze Biologiche*, 1928, **11**, 102, and *Science*, 1928, **67**, 593.

[3] Kindly furnished by the U. S. Bureau of Standards, Washington.

[4] American Rolling Mill Co. This iron contains 99.84% iron and less than 0.02% carbon, according to analyses furnished by the Company.

a single localized region must be present in which the reaction of the metal with the acid is continuous; this permanently active region may be compared with the nodal or pace-making region of the heart; it furnishes a constant activating influence to which the rest of the wire responds as soon as it has recovered sufficiently. From this controlling region waves of activation travel along the wire at intervals determined by the duration of the non-transmissive or "refractory" period which immediately follows each activation. In the rhythmically reacting wire, as in the heart, the regularity of the rhythm thus depends on the uniformity of the process of recovery, while the coördination or synchronization of activity in the different regions depends on transmission from a central or controlling region.

Since activation and transmission in passive iron are known to depend on the alteration or breakdown[5] of the passivating surface film by local cathodic reduction, it is clear that the special properties of the film differ in different kinds of iron and are determined, in some manner not yet understood, by the special composition of the metallic surface. The film formed on steel wires during repassivation is highly resistant to reduction by the current of the local active-passive circuit; hence transmission of activation for more than a short distance is at first impossible. It is only after some minutes (in 70 v.% HNO_3 at 20°) that the wire regains the ability to transmit activation waves to an indefinite distance.[6] On the other hand, in Armco wire and electrolytic wire, under precisely similar conditions, complete transmission returns within one second or less.[2] From evidence not yet published (having reference to the rates of recovery in different strengths of acid) it seems probable that the film formed on steel wire is at first relatively thick, and that recovery is the result of its progressive thinning by the solvent action of the acid, a certain limiting thickness (probably monomolecular) being eventually reached, corresponding to the final state of maximum transmissivity. In pure iron wire the passivating film is from the first thin; hence recovery is rapid. The influences of temperature and concentration of acid on the rate of recovery in Armco wire are similar in their general features

[5] Theoretically, a sufficient interruption of continuity is all that is required.

[6] Lillie, R. S., *J. Gen. Physiol.*, 1920, **3**, 107; 1925, **7**, 473.

to those found in steel wire, but the absolute rate of the process is much greater.

The rhythm is most readily demonstrated as follows. A short length of clean bright Armco wire (*e.g.*, 2 mm. thick, 1 to several cm. long) is placed in a flat-bottom vessel containing nitric acid of 60 to 80 v.% concentration.[7] Usually a rhythmical reaction starts at once; the metallic surface shows at regular intervals an alternation between a steel bright and a dull lustre; on closer examination it is seen that the effervescence is confined to the dark periods, while during the bright periods the metal is non-reactive (passive). The number of cycles per minute varies usually between 40 and 100 or more, increasing with the concentration of acid and the temperature. The relative duration of the passive phase in each cycle increases with the strength of acid; in the weaker acid the appearance is that of a bright flash passing at intervals over the dark effervescent surface of the metal. If the acid be left unstirred, the rhythm soon becomes irregular and passes over into a continuous effervescence; but with stirring a regular rhythm may last indefinitely, *i.e.*, until most of the wire is dissolved.[8]

Experiment shows that under these conditions the presence of the continually active or controlling region depends on the contact of the metal with the glass. At some local area of contact the diffusion of reaction products and acid is retarded and the acid soon becomes too weak to repassivate the iron; the reaction then becomes continuous, *i.e.*, a permanently active or anodal area is established. A wire freely suspended in acid by thin glass filaments is non-rhythmical; when activated it shows a single reaction and immediately becomes again passive and remains so. Rhythm, however, can readily be induced by locally interfering with diffusion or otherwise maintaining a permanent local activity.[9] The method finally adopted was to insert one end of the suspended wire into the interior of a narrow glass tube for a few millimeters. When such a wire is activated a few times (by touching

[7] Volumes of HNO_3, C. P., Sp. Gr. 1.42, in 100 volumes of solution.

[8] For further details *cf.* Lillie, R. S., *loc. cit.*[3]

[9] *E.g.*, continual contact of copper, zinc or other metal, anodal in relation to passive iron, will induce rhythm; but such rhythms are irregular because of the difficulty of maintaining constant conditions.

with zinc) continuous effervescence soon appears in the enclosed region, and waves of activity then pass at regular intervals from this region along the whole wire. When the acid is stirred sufficiently to render the local conditions stationary, the rhythm preserves its regularity; otherwise it fluctuates and tends to accelerate. Withdrawing the wire from the tube or otherwise arresting activity in the the pace-making area (*e.g.*, by contact with platinum) at once arrests the rhythm, and the wire becomes permanently passive, Conversely, increasing the active area, as by inserting the wire farther into the tube, accelerates the rhythm.

The manner in which the rhythm is influenced by the extent of the controlling active area, temperature, concentration of acid, electrical polarization of the wire by an external current, and length of wire have been briefly described in the two papers already cited.[9a] In the present paper the electromotor variations accompanying the rhythm will be described in some detail and their chief resemblances to the biological electromotor rhythms indicated. The variations of reaction velocity in the pulsating wire are an index of parallel variations in the passivating surface film, a regular rhythm implying alternate formation and breakdown of the film. The electrical variation is the most convenient and sensitive indicator of these changes in the film. Since there is evidence that variations in the semi-permeable protoplasmic surface films, affecting their permeability and electromotor properties, determine the bioelectric variations (membrane theory of Ostwald, Bernstein and their successors), the parallels between the electromotor variations in the two types of system are of interest as indicating the nature of the fundamental conditions determining activity in the irritable living system. In both cases the variations in chemical and electromotor activity appear to be primarily dependent on variations in the structure and composition of interfacial films.

The wire (Armco, *ca.* 2 mm. in diameter) was bent so that a straight portion of its length, *e.g.* 6 cm. long, could be immersed horizontally

[9a] Another form of rhythmical action is seen in "circuit transmission" in which a single "trapped" activation wave travels continually round and round a circular wire immersed in 80 v.% HNO_3. Each region of such a wire shows a regular rhythm determined by the rate of travel of the wave. For a description of this phenomenon *cf.* R. S. Lillie, *Science*, 1929, **69**, 305.

in the acid, which was contained in a vessel 10 cm. in diameter provided with an outflow tube inserted into its side 1.5 cm. above the bottom. The other end of the wire was attached to a key (Harvard cross circuit type) from which connection was made through a rheostat to the string galvanometer. The other electrode was a strip of platinum foil placed parallel to the wire at 2 to 3 cm. distance. A short portion of the wire, including the bend where it passed from the acid into the air, was coated with paraffin (to prevent irregular action which otherwise occurs at this region), leaving a length of *ca.* 5 cm. exposed to the acid. Opposite the free end of the wire was placed a glass tube, of calibre slightly greater than the diameter of the wire, into which the latter could be inserted for a short distance. In these experiments the inserted length was adjusted so as to secure the slowest rhythm that was stable under the conditions. The platinum electrode and the wire were connected with the terminals of a tube rheostat of low resistance (29 ohms), and wires led from the sliding contact and one terminal to the string galvanometer, a small instrument of the permanent magnet type. The tension of the string and the distance between slide and terminal of the rheostat were kept constant throughout the experiments. The speed of the recording surface was also constant at 0.8 cm. per second. The acid was led into the vessel by a siphon with its outlet near the insertion of the wire into the glass tube. The slow flow of acid provided the necessary stirring.

The records contained in the present paper were made at room temperature (20° to 22°). Under constant external conditions, with rhythms satisfactorily uniform, different wires show some variation in the rate of rhythm and in the form of the curve shown in each single cycle, but the essential features of the phenomenon are remarkably constant. The rate of rhythm increases with increase in the concentration of acid between 60 and 80 v.%. On either side of this range regular rhythms are difficult to obtain; at 55 v.% the automatic return of passivity is uncertain and irregular, while at 80 v.% and higher the tendency to passivity predominates and the active period becomes extremely brief. The rhythms at 80 v.% are typically rapid, broken and irregular (Fig. 1). At 85 v.% no satisfactory rhythms could be obtained.

In a large number of observations the rhythms observed in the

different concentrations of acid at 20–22° fell nearly always within the following ranges:

Concentration (v. % HNO_3)	*Rate per minute*
60	30–40
65	45–60
70	60–80
75	85–100
80	120 or higher

An approach to a linear relation between rate and concentration seems indicated. The velocity and extent of the reaction in the pace-making area determine the rate at which iron ions pass into solution, and hence the intensity of the local activating current; and by the mass action law the reaction velocity should be proportional to the concentration of acid. But the area of the permanently active region cannot be controlled with any exactitude by the simple method used; it also varies as the reaction proceeds and the wire is dissolved away. There is also a rise of temperature in the enclosed column of acid, and this condition is probably largely responsible for the rapid acceleration of rhythm at the higher concentrations.

Of greater interest is the manner in which the form of the curve varies in the different solutions. Figs. 1 to 5 give typical examples of records obtained from short lengths of Armco wire under the conditions described. The concentrations of acid were respectively 80, 75, 70, 65 and 60 v.%. In these curves the upstroke corresponds to the change from passive to active, *i.e.*, the iron becomes more anodal. It is apparent that as the concentration falls the duration of the active phase increases; activity is also more readily maintained at a nearly constant level, *i.e.*, the tendency to passivity decreases. The plateau signifies a temporary maintenance of the active state; this feature of the curve becomes more pronounced with decrease in concentration. With still further decrease the active phase lengthens rapidly and becomes indefinitely prolonged at a concentration of *ca.* 55 v.%; *i.e.*, no spontaneous repassivation occurs in acid below this concentration. It is also noteworthy that the "dip" of the curve, representing the range of the variation of potential, is maximal at an intermediate concentration of 65 to 70 v.%. The period of steel-bright lustre is very brief in 60 v.% acid, as already mentioned, and the slightness of

the dip in this solution indicates that passivity is less completely attained than in stronger acid; this apparently signifies that the temporarily formed film covers a smaller portion of the surface.

In all cases the upstroke, signifying the breakdown of the passivating film, is rapid, reaching a maximum within a small fraction of a second. The process of repassivation, corresponding to the reformation of the film, then begins immediately, as indicated by the downward slope of the curve; this slope is steep and almost uniform in 80% acid and becomes more gradual as concentration decreases. The rather definite turning point or inflection in the downward slope, seen especially in the weaker acid, indicates that the passivation process undergoes rapid acceleration after reaching a certain critical stage. This stage may be taken as corresponding to the formation of a definite area of film-covered, *i.e.* cathodal, surface. Theoretical considerations indicate that as the cathodal area increases the total electric current between the active and the passive portions of the metallic surface also increases progressively up to a maximum;[10] and it is probable

[10] This current, other conditions being equal, would be maximal with equal areas of anode and cathode, since in general the strength of current in a battery system is directly proportional to the area of each electrode, *i.e.*, to the product of anodal and cathodal areas. If the area of the whole surface of the wire is unity, and if we regard anodal and cathodal areas as sharply defined, then if $\frac{1}{n}$ is the cathodal area, $\frac{n-1}{n}$ is the anodal area. The product of the two, $\frac{n-1}{n^2}$, is maximal when $n = 2$, *i.e.*, when the wire is half covered with the film.

It is probable, however, that the *density* (*i.e.*, intensity ÷ electrode area) of the local current traversing the surface of the wire, rather than its total intensity, is the chief factor to be considered, since the density determines the rate of electrochemical reduction or oxidation in each unit area of surface and hence the rate of the activating or passivating influence at the region concerned.

Designating the electrode areas as above, we see that over the cathodal surface the average current density is $\frac{n-1}{n^2} \div \frac{1}{n} = \frac{n-1}{n}$; over the anodal surface it is $\frac{n-1}{n^2} \div \frac{n-1}{n} = \frac{1}{n}$. That is, the average current density at any time over either electrode surface is directly proportional to the area of the other electrode surface. This shows, *e.g.*, that the density of the current at the cathodal (*i.e.* passive) surface increases linearly as the anodal surface increases, and *vice versa*. Activation

that the increase in the rate of passivation (*i.e.* of film formation over the previously active areas) corresponds to a critical increase in the intensity or density of this current. Anodal oxidation, recognized as a chief general means of inducing passivity in metals, is undoubtedly a main factor in the reformation of the passivating film.[11]

In addition to these larger rhythmical variations of potential slight irregular fluctuations occur under a variety of conditions. In curves with well defined plateau (*e.g.* Figs. 4 and 5) a slight notch or dip is often seen just before the final rapid descent which accompanies repassivation. When the active phase is greatly prolonged, as in acid between 55 and 60 v.% concentration, an irregular rhythmical fluctuation often lasts throughout the entire active period. Similar variations frequently occur during the passive phase, especially when the "pause" is prolonged. Figs. 6 and 7 illustrate this condition. In the case illustrated by Fig. 6 the wire spontaneously ceased activity after a series of regular pulsations in 65 v.% acid (at 22°), apparently as a result of the dissolving away of the free end inside the glass tube. The last few beats show retardation with an accompanying increase in the range of variation; the potential then falls progressively, with irregular fluctuations indicating an oscillation between activating and passivating influences, to a level corresponding to complete passivity. In general, when the interval between successive cycles of a rhythmical series is prolonged, the variation of potential is increased;

requires more than a certain critical density of current; hence slightly scraping a passive wire in HNO_3 with glass may fail to activate the wire as a whole—*i.e.*, fail to cause a transmitted effect—while a larger scrape activates.[6] Correspondingly, any local active area in a passive wire will spread, with an automatic acceleration (*i.e.* explosively), as soon as the area becomes large enough; similarly (although the conditions differ in detail) with a passive area. The steepness in both the ascent and descent of the curves may thus be understood. The irregular oscillations about a midway position shown in Figs. 6 and 7 are also probably an expression of the mutual influence exerted by local anodal and cathodal areas. The existence of such an influence between the active and passive areas of the same wire was early noted by Schönbein (Philos. Mag., 1836, Vol. 9).

[11] This influence is seen (*e.g.*) in the retardation and arrest of the rhythm of a pulsating wire when it is made the anode of an external circuit.[2] The relative duration of the passive phase is at first lengthened, and with sufficient polarizing current the wire soon comes to rest in the passive state.

the form of the curve also undergoes a change which is illustrated in Figs. 6 and 7.

It will be seen from Fig. 6 that the whole variation of potential between activity and complete passivity in 60 v.% acid has approximately twice the range of that shown in each single cycle. The potential difference between completely active and completely passive wires, in 60 to 70 v.% HNO_3, as shown when the two are connected through a voltmeter, is of the order of 0.7 volt. During the rhythmical series the variation is *ca.* 0.35 volt, *i.e.*, the wire is reactivated before passivation has reached the complete or equilibrium stage. That this is the case is also shown by comparison with Fig. 8 which gives records of single variations resulting from successive brief contacts of zinc with a non-rhythmical wire in 65 v.% acid at intervals of a few seconds (22°). The variations are seen to be uniform in character and duration, but of approximately twice the range characteristic of each cycle of the rhythmical series.

In the comparison with living tissues the more general conditions are of chief interest and may be here briefly reviewed. The known relations between stimulation and variation of permeability indicate that alternate breakdown and reformation of interfacial films are the controlling factors in the living as well as in the non-living system, stimulation corresponding to breakdown of the protoplasmic surface film and recovery to its reformation. In both systems these processes are under electrical control. It is also to be noted that in general the recovery in living tissues is more directly associated with consumption of oxygen than is the stimulation; this would indicate that the normal semi-permeable properties of the film in the resting or recovered cell are determined by some oxidation product or products, or by some by-product of an oxidative reaction. Alternate oxidation and reduction are the essential features of the chemical reaction cycle in the passive iron system; and such general physiological facts as polar stimulation and electrotonus indicate that in this respect also the general conditions in irritable protoplasmic systems are similar. The complete dissimilarity in the chemical details of the controlling surface reactions in the two cases is in no sense incompatible with an identity in the fundamental physical conditions of the reactions.

The precise form of the electromotor curve in the rhythmical iron system shows considerable variation, as the foregoing examples illustrate. Similarly the form of the electrocardiogram varies widely in different animals;[12] these variations are to be referred in part to special anatomical peculiarities, as well as to differences in the time-relations and other features of the characteristic reaction cycles. In simple invertebrate hearts curves resembling closely those of the iron wires occur frequently. A rapid upstroke, sloping plateau and rapid downstroke are seen, for example, in the hearts of mollusca (Aplysia[13]) and Crustaceans (Maja,[13] Homarus[14]). Oscillations in the plateau are a regular feature of the hearts of Maja and Limulus.[13] In the vertebrate heart, with its several chambers and differentiation of conducting and contractile tissues, such resemblances are less evident, and variations in the position of the leading-off electrodes affect greatly the form of the curve. In many cases, however, the electromotor variation of a single chamber (*e.g.* ventricle) conforms to this type of curve.[15]

SUMMARY

1. The electromotor variations of pure iron wires, arranged to react rhythmically with nitric acid, are recorded and described.

2. Resemblances between these variations and those of rhythmically reacting living tissues (especially the heart) are pointed out and discussed.

EXPLANATION OF PLATES

PLATE 1

Figs. 1–5. Tracings from wires in HNO_3 of the concentrations 80, 75, 70, 65 and 60 v.%. Temperature 22°. The respective rates per minute are 140–150, 96–98, 60, 46 and 35.

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[12] For a review *cf.* the article by W. Einthoven, Handbuch der normalen und pathologischen Physiologie, 1928, **8**, 785.

[13] Hoffmann, P., *Archiv f. Anat. u. Physiol., Physiol. Abth.*, 1911, 135; *cf.* Fig. 14, Taf. X. *Cf.* also the tracings from the heart of Helix in the article of C. Lovatt Evans, *Zeitschr. Biol.*, 1912, **59**, 397, Fig. 3 and Plate VI, *A*, *B*, *D*.

[14] Hogben, L., *Quart. J. Exper. Physiol.*, 1925, **15**, 264; *cf.* Figs. 21, 22.

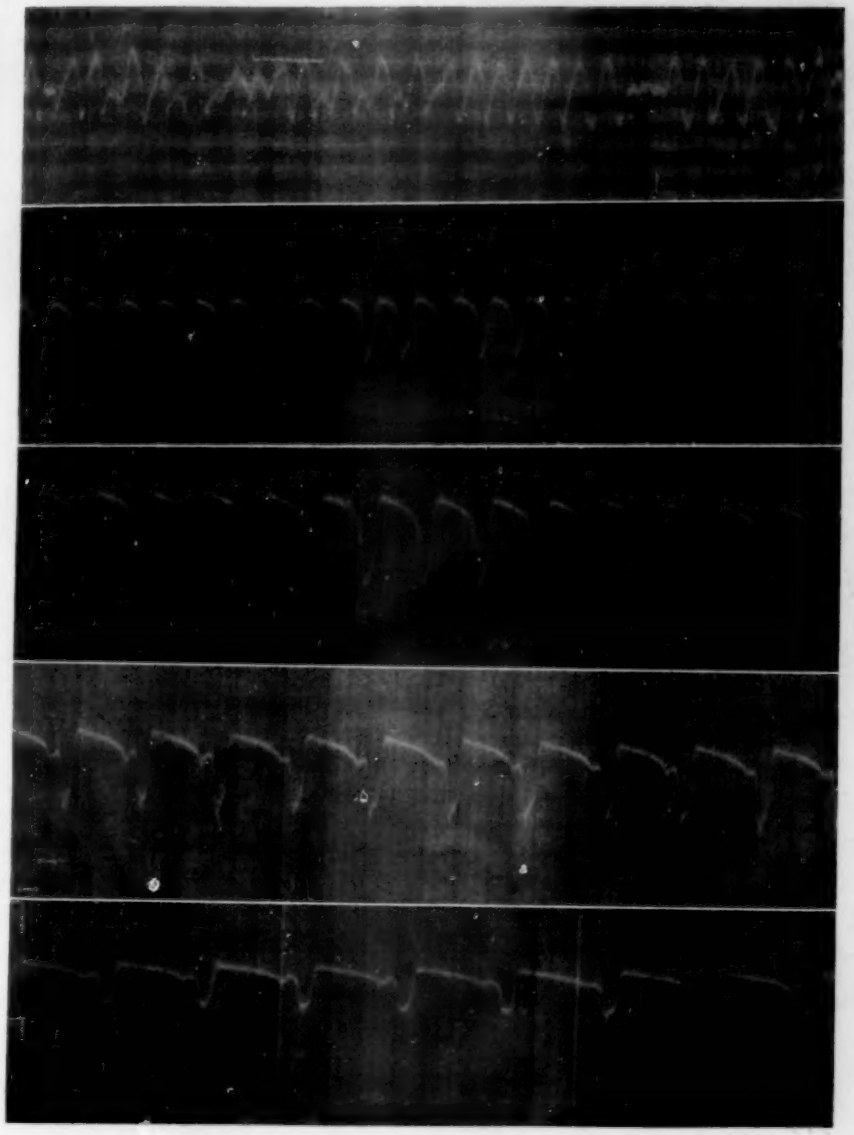
[15] *Cf.* the tracings from the frog's heart in Einthoven's article,[12] pp. 813, 849.

PLATE 2

Fig. 6. Tracing from wire in 65 v.% HNO_3 showing relapse from rhythmical to permanently passive state at the end of a series of pulsations. Note rapid oscillations of potential during the relapse. Temperature 22°–23°.

Fig. 7. Another part of the previous tracing showing prolonged pause with oscillations of potential between pulsations.

Fig. 8. Variations resulting from successive contacts of a non-rhythmical wire with zinc. HNO_3 65 v.%. Temperature 22°.



(Lillie: Resemblances between electromotor variations)

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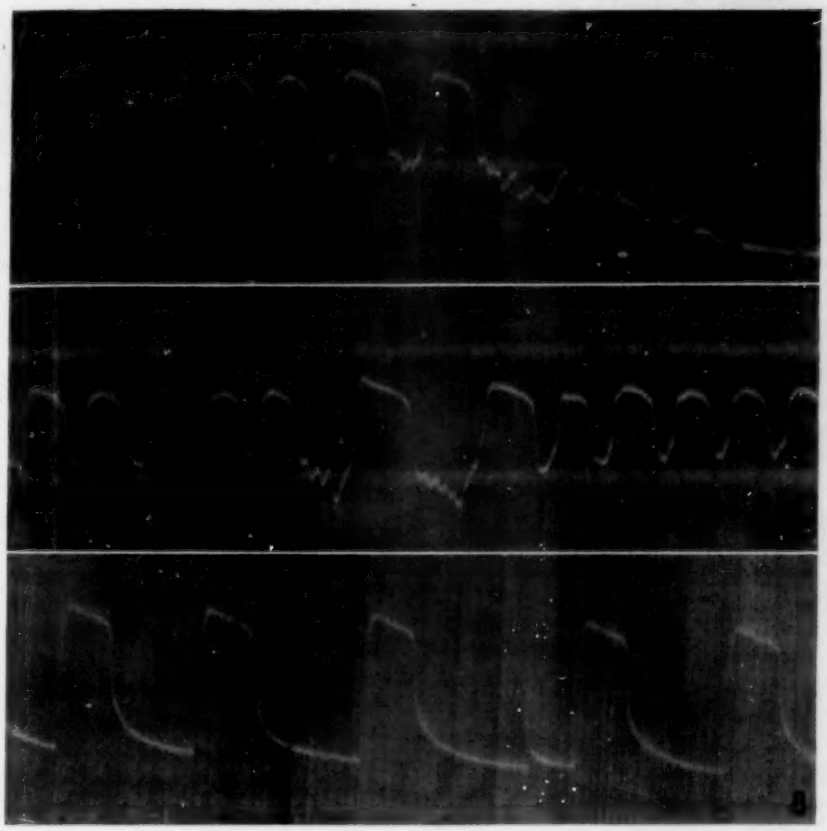

(Lillie: Resemblances between electromotor variations)

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A PRELIMINARY STUDY OF THE REDUCING INTENSITY OF LUMINOUS BACTERIA

By E. NEWTON HARVEY

(From the Physiological Laboratory, Princeton University, Princeton)

(Accepted for publication, May 22, 1929)

The studies of Clark[1] and his co-workers, and of Conant,[2] have called attention to the significance of oxidation-reduction potential as a measure of the reduction intensity of a chemical system, comparable to acidity. The investigations of Gillespie,[3] Cannan, Cohen, and Clark,[4] Coulter[4a] and Dubos[5] have shown the significance of the concept for cell suspensions, while the work of the Needhams,[6] Rapkine and Wurmser,[7] and Cohen, Chambers, and Reznikoff[8] has been concerned with the interior of the cell.

Briefly, it has been observed that different cells or cell suspension in absence of oxygen, possess a reduction intensity constant at a certain level, depending on the type of cell, which can be determined by ability to reduce some but not others of a series of oxidation-reduction (redox) indicators of different reduction intensity. When gold or platinum electrodes are placed in such suspensions the final electrical potentials obtained correspond to those expected from the redox indicator series. Some anaerobes may carry the potential to the overvoltage side of the hydrogen electrode and actually form hydro-

[1] Clark, W. M., and collaborators, Collected Studies, 1923–26, Bull. No. 151 Hygienic Lab. U. S. Pub. Health Service, *Chem. Rev.*, **2**, 127, 1925.

[2] Conant, J. B., *Chem. Rev.*, **3**, 1, 1926.

[3] Gillespie, L. J., *Soil Science*, **9**, 199, 1920.

[4] Cannan, Cohen and Clark, Supp. No. 55 to Pub. Health Reports, 1926.

[4a] Coulter, C. B., *J. Gen. Physiol.*, **12**, 139, 1928; Coulter and Isaacs, *J. Exp. Med.*, **49**, 711, 1929.

[5] Dubos, R., *J. Exp. Med.*, **49**, 507, 559, 575, 1929.

[6] Needham and Needham, *Proc. Roy Soc.*, **98**, 259; **99**, 383, 1926; *Protoplasma*, **1**, 255, 1926.

[7] Rapkine and Wurmser, *Proc. Roy Soc.*, **102**, 127, 1926.

[8] Cohen, Chambers and Reznikoff, *J. Gen. Physiol.*, **11**, 585, 1928.

gen. In aerated suspensions the observed potential is naturally much further toward the oxidizing side and correspondingly redox indicators cannot be reduced that reduce easily under anaerobic conditions, but even so both cells and cell suspensions possess far greater reducing intensity than would correspond to the air electrode and indicators are held reduced that oxidize readily in air. Both suspensions and cells are rather poorly poised, but new reducing material seems to be slowly mobilized from some large reserve. Perhaps it should be emphasized that the above statements apply when the redox indicator is injected into a cell as well as when cell suspensions in the redox indicator solutions are used.

Luminous bacteria offer special advantages for oxidation-reduction studies, since the luminescence is an indication that oxidative reactions connected with light production are proceeding within the cell. In the crustacean, *Cypridina*, luminescence is connected with the oxidation of luciferin to oxyluciferin in presence of luciferase. *Cypridina* luciferin can only be oxidized to oxyluciferin rapidly by such systems as quinhydrone (not by o-chloro-indophenol) while oxyluciferin can only be rapidly reduced by such systems as anthraquinone 2, 6 di Na sulphonate. Between these systems luciferin is not oxidized by an oxidant and oxyluciferin not reduced by a reductant, so that we are not dealing with a truly reversible equilibrium and I have spoken of the apparent oxidation and apparent reduction potential of luciferin-oxyluciferin in Conant's[8] sense (Harvey[9]).

Luciferin and luciferase have never been demonstrated surely in luminous bacteria and the luminescence of these forms seems to be connected closely with other oxidative processes in the bacteria, since it is dimmed by dilute KCN which has practically no effect on the luminescence intensity of *Cypridina*. It should be pointed out, however, that KCN has a far greater effect in suppressing the respiratory oxidations than in decreasing bacterial luminescence (Harvey[10]) and Shoup[11] has recently shown that the oxygen consumption can be reduced to one-half the normal value by low concentrations of oxygen

[9] Harvey, E. N., *J. Gen. Physiol.*, 10, 385, 1927.
[10] Harvey, E. N., *J. Gen. Physiol.*, 8, 89, 1925.
[11] Shoup, C. S., unpublished observations.

before the luminescence intensity is affected. The respiratory and luminescence oxidations are not equally affected.

Nevertheless there are certain phenomena connected with the luminescence of bacteria as affected by the redox indicators, that suggest rapid oxidation of luciferin, although the interpretation of effects on luminescence are difficult. These will be considered below.

These oxidation-reduction studies were carried out on a suspension of the bacterium, a rod-shaped form isolated from fish and identified as *Bacillus fischeri* by Dr. S. E. Hill,[12] in M/4 phosphate buffer of P_H = 7.6, in which respiration and luminescence continue for some hours. If allowed to stand they use up the oxygen, when their luminescence disappears, and produce CO_2 in corresponding amount, but an amount which is too small to change the P_H of the highly buffered medium. The oxidized indicator[13] solutions were made up fresh and placed in tubes in small amount and sufficient thoroughly aerated concentrated bacterial suspension added to bring the indicator concentration to approximately M/100,000 to M/10,000. No doubt the proper method of comparing redox indicators is in equimolecular concentration but some indicators are so highly colored as compared with others in equimolecular concentration that the color obscures the luminescence.[14] I have found it better in practice to add enough indicator to give just observable color that does not absorb much of the light from the baceria. It must be borne in mind that too much indicator added to bacterial suspension will overbalance the reducing capacity of the bacteria and no reduction of the indicator will occur even though plenty of time is allowed. The luminescence will also be permanently quenched by higher concentrations of indicators.

The mixture of indicator and bacterial suspension then stood undisturbed and the effect on luminescence and on color change in the indicator noted, especially the relation between reduction of the indicator and disappearance of luminescence. Part of the time for

[12] Hill, S. E., Biol. Bull., **55,** 143, 1928.

[13] Most of the indicators were obtained from the La Motte Chemical Products Company. I am deeply indebted to Dr. Barnett Cohen for Nos. 5, 6, 13 and 17, and to Dr. J. B. Conant for Nos. 9, 10, 14, 15, 21, and 22.

[14] Quinone has a far greater effect in dimming a suspension of luminous bacteria than o-Cl-indophenol in the same molecular concentration.

disappearance of color is that necessary for the bacteria to use up the oxygen in solution and part, the time required to reduce the dye. Of course luminescence always disappears when dissolved oxygen has been utilized by the respiration.

Observations were also made on the intensity of luminescence and the reoxidation of the reduced dye by air, when the suspension is again thoroughly aerated. Table I gives these general relations for a series of reversible indicator and oxidation-reduction systems. Some are known to be relatively harmless for sea urchin eggs[8] (Column 7) and present no obvious toxic effects on luminous bacteria (Column 8). Others are definitely toxic and for this reason it is somewhat difficult to analyze their effects.

Considering first Column 5, it is generally true that reduction is easiest the higher the indicators stand in the series. This is shown by the time for reduction, which in general lengthens as we proceed downward in the series and especially by the fact that some of the dyes can be reduced before the luminescence disappears or dims, others reduce simultaneously with the dimming of luminescence, and others only after all trace of luminescence has disappeared. The indigo mono-sulphonate cannot be reduced at all. These relations are indicated by the letters a, b, c, which come in order from above down.

Shoup's studies[11] in this laboratory have indicated that the luminescence of an emulsion of bacteria just begins to dim at about 0.26 per cent oxygen (2.6×10^{-3} atmosphere). At oxygen pressures above this value the luminescence intensity remains the same. A just perceptible luminescence can be detected with an oxygen pressure of 0.0007 per cent (7×10^{-6} atmosphere) (Harvey and Morrison[15]) but this value is only to be regarded as an order of magnitude, since it depends on many variable factors such as dark adaptation of the eye, thickness and concentration of bacterial emulsion, etc. The reduction of some indophenols while the luminescence is still bright indicates that there must be present in the emulsion 0.26 per cent oxygen, an amount far above the theoretical pressure of oxygen that would be in equilibrium with an electrode in contact with reduced indophenol. The observation simply shows that the reduction of

[15] Harvey and Morrison, *J. Gen. Physiol.*, 6, 13, 1923.

TABLE I

	Molecular weight	E_O at $P_H = 7$	R_H	R_O	Color change	Luminescence	Toxicity to *Amoeba dubia*	Toxicity to luminous bacteria
O_2 electrode		+0.810	41.0	0				
Air electrode		+0.80?	40.7					
1. $K_3Fe(CN)_6$	329.2	+0.430	28.4			2		N
2. Quinone	108.0	+0.278				1		T
3. o-Cl indophenol	255.6	+0.233	21.8	38.5	ax	1′	N	N
4. Phenol indophenol	221.0	+0.227	21.6		ax	1′	N	N
5. Bindschedler's green, base	256.0	+0.224	21.5		ay	1	T	T
6. 2,6 di Cl indophenol	290.0	+0.217	21.3		ax	1	N	S (?)
7. o-cresol indophenol	235.0	+0.194	20.5	41.0	ab xy	1′	N	N
8. 2,6 di Cl 3 methyl indophenol	305.0	+0.181	20.1		ab xy	1	S	S
9. p-xyloquinone	136.0	+0.170				1		T
10. 1,2 napthoquinone	158.0	+0.127				1		T
11. 1 napthol 2 SO_4 indophenol	373.0	+0.123	18.1		ab y	2	S	N
12. 1 napthol 2 SO_4 indo 2,6 di Cl phenol		+0.119	18.0		by	2	T	N
13. Toluylene blue chloride H_2O	308.0	+0.115	17.9		by	1	S	S (?)
14. 2 Cl 1, 4 napthoquinone	227.0	+0.080				1		T
15. 1,4 napthoquinone	158.0	+0.070				1		T
16. Methylene blue Cl $5H_2O$	410.0	+0.011	14.4		by	2	S	N
17. K_4 indigotetrasulphonate	734.0	−0.046	12.5		by	2	T*	N
18. K_3 indigotrisulphonate		−0.081	11.3		by	2	T*	N
19. K_2 indigodisulphonate	498.0	−0.125	9.9		by	2	T	N
20. K indigosulphonate	380.0	−0.182	7.5	63.0	c	2		N
21. Na_2 anthraquinone 2,6 disulphonate		−0.192				2		N
22. Na anthraquinone 2 sulphonate		−0.233				2		N
H_2 electrode		−0.421	0	82.0				

a. Color fades before luminescence disappears. Reduction.
b. Color fades after luminescence disappears. Reduction.
c. Color does not disappear. No reduction.
x. Color disappears while air bubbled through; no return on shaking with air.
y. Color remains while air bubbled through; returns on shaking with air.
1. Luminescence progressively dims.
1′. Luminescence dims at first but returns again.
2. Luminescence not dimmed until oxygen used up.

Toxicity for *Amoeba* after Cohen, Chambers and Reznikoff.[8] Toxicity for bacteria judged by progressive dimming and failure of light to return on shaking thoroughly with air. T, toxic; T*, oxidant toxic, reductant non-toxic; S, slightly toxic; N, non-toxic.

indophenol in the bacterial suspension is no indication that the oxygen has been reduced to a very low figure and it is quite possible that in suspensions of bacteria containing reduced methylene blue, even though no luminescence is visible, the concentration of oxygen is not so low that the "last molecule" has disappeared, *i.e.*, the oxygen pressure may not necessarily be that theoretically in equilibrium with an electrode in contact with reduced methylene blue. The R_o values given in the table are $\log \frac{1}{P_o}$ values where P_o = oxygen pressure in atmospheres. The oxygen pressure in the case of o-indophenol would be theoretically $10^{-36.5}$ atmosphere, whereas the bacteria are still luminescing brightly.

From the data in the table we can put the reduction potential of the bacterial suspension in air in the R_H region 18 to 20 whereas the reduction potential in absence of oxygen is in the region $R_H = 8$ to 10. We might predict that potentiometric observation with the gold electrode in nitrogen should give an E_H value around -0.20 volts or a potential of -0.446 against the saturated KCl calomel electrode. One of my students, Mr. Emerson Holcomb, is now engaged in a study of these potentials and a quantitative investigation of the reduction of these systems, penetration, the place where reduction occurs, whether inside or outside the cell, etc.

The reduction of a number of other dyes whose position in the redox scale is not accurately known was also tested, namely: trypan red, alizarin blue S, Na carminate, bismark brown, neutral red, trypan blue, anilin green, brilliant cresyl blue, bordeaux red, toluidine blue, janus blue, methyl violet B, thionin, isamine blue, methyl green, diazin green, rhodamine, vital red HR, neutral fuchsine, nile blue and safranin. Of these, only brilliant cresyl blue, toluidine blue, and thionin were reduced.

These luminous bacteria are obligatory aerobes (Shoup[16]) and their behavior toward indicators is what we should expect from our general knowledge of the behavior of aerobic cells and suspension toward the redox indicator series.[4] The luminescence does not endow them with any unusual behavior.

[16] Shoup, C. S., *Proc. Soc. Exp. Biol. Med.*, **25**, 570, 1928.

When effects of the redox series on luminescence are analyzed we can make the general statement that the more oxidizing members readily dim the luminescence while the more reducing members (in the oxidant form) do not, apart from the absorption of light which results from their color. One might interpret this as meaning that their redox potential is greater than that of luciferin and consequently they oxidize most of the luciferin, leaving none or little for luminescence. Such an interpretation is attractive and from what we know of the behavior of *Cypridina* luciferin quite applicable to quinone, but in view of the fact that 1.4 napthoquinone, which stands low in the series, also causes rapid dimming as well as other napthoquinones and xyloquinone, we cannot regard this explanation as unequivocal. It may merely be that the quinones in general are toxic, for the luminescence of bacteria can be dimmed by anesthetics and other substances which have no oxidizing action. However, the anthraquinone sulphonates (in oxidized form) do not affect the luminescence and also stand well down in the series.

One might expect that K ferricyanide, a strong oxidizing substance and hence one which should immediately oxidize luciferin, would dim the light and yet there is no effect with either K or NH_4 ferricyanide, even in M/1000 concentration. I interpret the lack of effect to mean that these salts cannot penetrate the bacterial cell. $KMnO_4$ dims somewhat in M/5000 concentration, whereas quinone prevents luminescence entirely at far greater dilutions than this.

The chief interest lies in systems of which o-Cl-indophenol is a good example, which, in weak concentrations, cause an initial dimming of the luminescence which soon returns to its original brightness. This recovery of the luminescence intensity occurs as the color of the indicator fades but is not entirely connected with absorption of light, as dimming occurs in concentrations so weak as to have practically no color. I interpret it to mean a rapid oxidation of luciferin by the indophenol so that no luciferin is left in the cell to produce light. As the indophenols are reduced more luciferin can be produced and the luminescence returns. The dyes are reduced while the luminescence is bright, showing that oxygen is present, and in fact the dyes can be reduced while air is bubbled through the suspensions as has been observed with many indophenols.[4] Addition of ferricyanide causes

immediate return of the dye color by reoxidation and a second dimming of luminescence. So long as we hold the indophenol in the oxidized form the luminescence is dimmed.

The indicator dyes having an R_H of 18.1 or less, with the possible exception of toluylene blue, do not seem to affect the luminescence in small amount apart from the absorption of light due to their color, and it is especially interesting to note that the colorless anthraquinones do not interfere with the luminescence, and should not from their position in the redox series.

SUMMARY

The effect of a series of redox indicators and systems has been tested with a suspension of luminous bacteria (*B. fischeri*) in M/4 phosphate buffer of $P_H = 7.6$.

The indicators behave as expected from their position in the redox series, the most positive being reduced rapidly even in presence of air and before luminescence of the bacteria disappears, those of intermediate position at the time luminescence disappears, and the more negative only long after the luminescence had ceased, due to utilization of oxygen by the bacterial respiration. Indigo monosulphonate was the only indicator not reduced on long standing of a bacterial suspension. The aerobic redox potential may be placed at an $R_H = 18–20$ and the anaerobic potential at an $R_H = 8–10$.

Ferricyanides do not affect luminescence and behave as if they could not penetrate the bacterial cell. Quinone and the napthoquinones cause progressive dimming of luminescence in any concentration which affects the light but it cannot be definitely stated that this is due to rapid oxidation of luciferin although it seems likely in the case of quinone. Some indophenols dim the luminescence at first, followed by return of brightness, which is interpreted to mean rapid oxidation of luciferin while the indophenol is unreduced, more luciferin production after reduction of indophenol. The more negative redox systems do not affect the luminescence. Investigation of indicator reduction and luminescence is being continued.

UNEQUAL DISTRIBUTION OF IONS IN A COLLODION CELL

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Collander (1) found that the permeability of "dry" collodion membranes was essentially the same as that of living cells in that they allowed the passage of weak acids, bases, etc., but not of salts nor strong acids. Living cells possess another striking characteristic in that they are able to maintain a constant difference in concentration of solutes between the interior of the cell and the surrounding solution. This behavior can be accounted for if it be assumed that the substance becomes changed after entering the cell to one which cannot pass through the membrane. The non-diffusible form will then concentrate inside the cell. The results obtained in the previous study (2) of dry collodion membranes predict the conditions necessary for this result.

Concentration of Iodide Ion

Dry collodion membranes are readily permeable to iodine but are impermeable to iodide ion or to thiosulphate. If, therefore, a saturated solution of iodine is separated from a solution of thiosulphate by such a membrane the iodine will diffuse through the membrane and become reduced to iodide which cannot diffuse out. The iodide will therefore continue to collect in the cell. This will continue until all the thiosulphate has been used up and the activity (vapor pressure) of the iodine becomes equal on the two sides of the membrane.

The result of an experiment set up in this way is shown in Fig. 1. The membranes were made as previously described and were suspended in 500 cc. of a saturated solution of iodine in water with excess solid iodine present. The concentration of iodine outside remained constant. The concentration of iodide inside increases slowly, and until about the 50th day no iodide was found outside showing that the mem-

brane is impermeable to both thiosulphate and iodide. At this time the membranes became yellow and crinkled and the concentration of iodide inside suddenly increased, while at the same time iodide began to appear in the outside solution. The membranes had evidently lost their semipermeability and become like dead cells.

Concentration of Chloride Ions

It was found previously that mercury chloride passed through dry collodion membranes while all other chlorides tested could not pass through. A system, therefore, in which the mercury chloride was

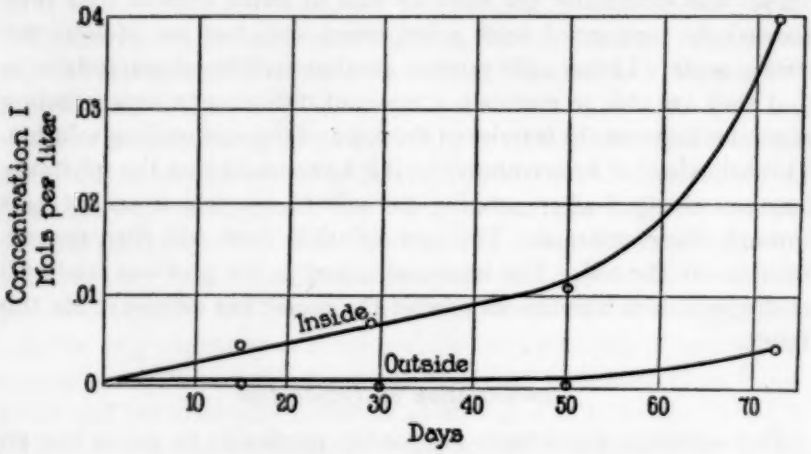

FIG. 1. Concentration of iodine in cell: $Na_2S_2O_3$ 0.10 M/I_2 — water.

changed to another chloride after entering the cell would concentrate chloride ions.

The results of such an experiment are shown in Fig. 2. The cell contained originally 0.011 M $HgCl_2$ and 0.05 M Na_2CrO_4 inside and 0.011 $HgCl_2$ and 0.05 Na_2SO_4 outside. On mixing the mercury was precipitated as $HgCrO_4$. The activity of the mercury chloride was therefore less inside than out and so more mercury chloride diffuses in and the chloride ion concentration increases as shown in the figure. This would continue until the product of the Hg and Cl ion activities inside and out were equal. Since the membrane is permeable for water it would also be necessary that the vapor pressure of the water

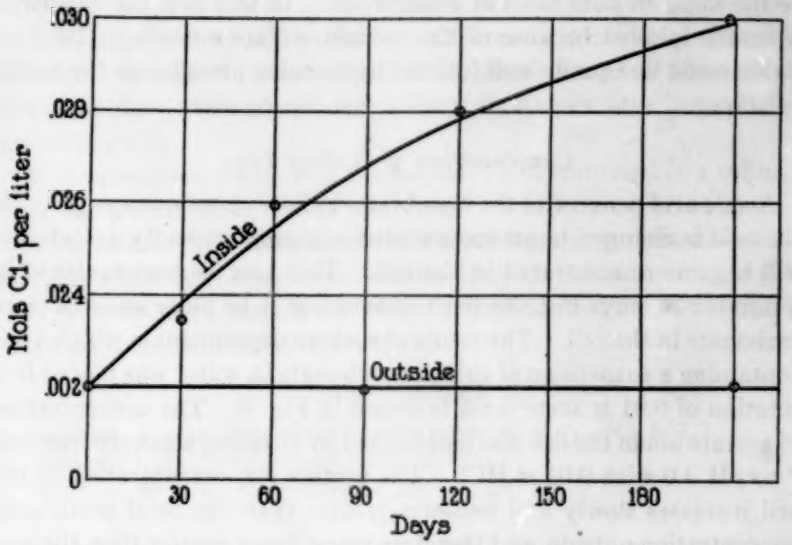

FIG. 2. Concentration of Cl^- ions in cell: 0.011 $HgCl_2$, 0.05 Na_2CrO_4/0.011 $HgCl_2$, 0.05 Na_2SO_4.

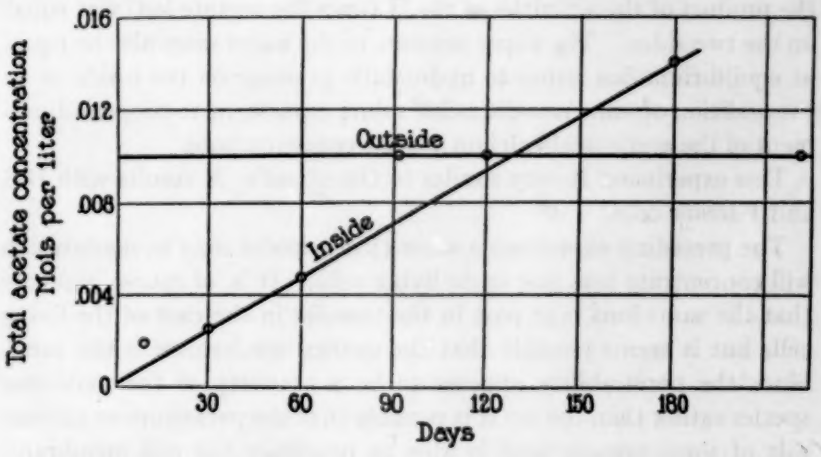

FIG. 3. Concentration of acetate ion in cell: 0.01 acetic acid/$CaCO_3$ — water.

be the same on both sides at equilibrium. In this case the condition is nearly fulfilled because of the sodium sulfate outside. The condition could be equally well fulfilled by exerting pressure on the inside solution.

Concentration of Acetate Ions

Acetic acid penetrates the membrane while acetate ion does not. If the acid is changed to an acetate after entering evidently acetate ion will become concentrated in the cell. This may be accomplished in a number of ways but the most convenient is to place solid calcium carbonate in the cell. The result of such an experiment in which a cell containing a suspension of calcium carbonate in water was placed in a solution of 0.01 M acetic acid is shown in Fig. 3. The concentration of acetate ion in the cell was determined by titrating a sample from pH 7 to pH 3.0 with 0.01 M HCl. The acetate ion concentration in the cell increases slowly and becomes greater than the total acetic acid concentration outside, and therefore many times greater than the acetate ion concentration outside. The process would continue presumably until the activity of the acetic acid (which is proportional to the product of the activities of the H times the acetate ion) was equal on the two sides. The vapor pressure of the water must also be equal at equilibrium due either to hydrostatic pressure on the inside or to the addition of some non-diffusible solute outside, or to proper adjustment of the acetic acid-calcium acetate concentrations.

This experiment is very similar to Osterhout's (3) results with H_2S and *Valonia* cells.

The preceding experiments show that a model may be made which will concentrate ions just as do living cells. It is, of course, unlikely that the same ions take part in the transfer in the case of the living cells but it seems possible that the general mechanism is the same. Since the permeability appears to be a property of the molecular species rather than the ion it is possible that the potassium or calcium salt of some organic acid is able to penetrate the cell membrane. Most organic acids are oxidized in the cell and if this occurred, the ion which entered with the organic ion could not escape and would be concentrated in the cell. There are evidently a number of other possibilities which would lead to the same result.

SUMMARY

The properties of dry collodion membranes previously described allow the prediction that cells of these membranes will concentrate solutes under certain conditions. Three such cases have been studied experimentally.

1. A membrane containing thiosulphate and immersed in a solution of iodine concentrates iodide ion.

2. A membrane containing sodium chromate and immersed in mercury chloride concentrates chloride ion.

3. A membrane containing calcium carbonate and immersed in acetic acid concentrates acetate ions.

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THE RESPIRATION OF LUMINOUS BACTERIA AND THE EFFECT OF OXYGEN TENSION UPON OXYGEN CONSUMPTION

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INTRODUCTION

In the case of large organisms the rate of respiratory exchange will depend (1) on the amount of oxygen available for use, and (2) on adequate respiratory and circulatory mechanisms in the body. Amberson and his associates[3] in the case of several marine invertebrates, and S. Nomura[22] with the Holothurian *Caudina*, have shown that the rate of oxygen consumption is proportional to the oxygen tension of the medium. Pütter[26a] examined the oxygen consumption of several invertebrates at low pressures of oxygen and determined the limiting value for oxygen at which respiratory activity was equivalent to that in air. Very recently Hall[10] has shown the dependence of certain marine fishes upon the amount of available oxygen in the medium and the haemoglobin content of the blood for complete respiration. In all of these larger forms the rate of oxygen consumption is dependent upon oxygen concentration over a wide range of oxygen pressures.

In general, organisms such as protozoa or bacteria are small enough to allow complete diffusion of gases dissolved in the medium, and consume oxygen at a constant rate independently of the oxygen pressure until the oxygen concentration falls to a point allowing incomplete activity of the respiratory mechanism within the cell. Only then does the rate of oxygen consumption diminish, and in many forms this limiting value for adequate respiration will be very small indeed, as has been shown by various workers, (Warburg,[24a] Henze,[13] Lund,[19] Pütter,[26b] Harvey [11b]) whose experiments indicate that in unicellular

organisms oxygen consumption remains independent of oxygen concentration over a very wide range of oxygen pressure. Amberson[2] has shown that the oxygen uptake of Paramecium is practically constant from 200 to 50 mm. Hg oxygen, and the limiting value for adequate respiration in these organisms is not reached until the oxygen tension falls below 50 mm. At 11 mm. of oxygen the reduction in the rate of respiration is only 20 per cent of that at atmospheric pressure. Lúnd[19] has obtained results in agreement with those of Amberson on the same organism. Amberson also conducted experiments on the dividing egg of the sea-urchin and found that respiratory activity does not fall away from the rate at atmospheric pressure of oxygen until 80 mm. Hg is reached, and no marked decrease in the rate of respiration occurs until oxygen pressure has been reduced to 20 mm. Hg.

Estimations have been made of the rate of oxygen consumption with abundant oxygen by luminous bacteria when suspended in seawater, by methods involving the time for dimming (Harvey[11b]) and by a manometric method (Harvey[11c]). In the present investigations measurements have been made of actual values for oxygen consumption and carbon dioxide production when the amount of available oxygen is diminished, and also the rate of respiratory exchange before and following the dimming of luminesence of luminous bacteria due to lack of oxygen. The amount of oxygen necessary for the maximum luminesence is less than that required for maximum metabolism of the cell, and luminesence will still occur when only a very small amount of oxygen is present (Beijerinck,[5] Harvey[11d]). The actual amount of oxygen necessary to give just visible luminesence has been estimated by Harvey and Morrison[11e] to be of the small value 0.0053 mm. Hg (0.0007 per cent). At least 90 per cent of available oxygen is consumed when dimming of a suspension of luminous bacteria occurs.

In these experiments oxygen consumption by luminous bacteria has been followed by two methods: (1) Colorimetric, involving the use of haemocyanin as an indicator of the presence of dissolved oxygen in suspensions of bacteria, and (2) a manometric method allowing direct volumetric determinations to be made of the amount of oxygen consumed in given time.

Colorimetric Determinations of Oxygen Consumption

It is necessary to have a convenient indicator for the presence of oxygen if the rate at which oxygen is removed from a suspension of bacteria is to be observed. No colorimetric indicator of oxygen is available in which living cells may be placed excepting the blood-serum of certain crustaceans containing the copper-protein compound haemocyanin. The methods followed in these experiments are similar to those suggested by Osterhout[23] and Harvey[11a] for the use of *Limulus* haemocyanin as an oxygen indicator. Luminous bacteria live perfectly well in the oxygenated *Limulus* serum, and as they consume oxygen from the serum in which they are suspended it passes from the dark blue of fully-oxygenated oxyhaemocyanin to the colorless reduced haemocyanin free of oxygen.

TABLE I

Color Standards

Tube No.	Oxygenated haemocyanin	
1	1 part to 9 parts sea-water	Each tube with 1 cc. bacterial suspension killed with toluol
2	2 parts " 8 " "	
3	3 " " 7 " "	
4	4 " " 6 " "	
5	5 " " 5 " "	
6	6 " " 4 " "	
7	7 " " 3 " "	
8	8 " " 2 " "	
9	9 " " 1 part sea-water	
10	10 " " 0 " "	

The color change of a standing tube of *Limulus* serum containing luminous bacteria was followed, and the actual time for the consumption of available oxygen was determined. The experimental tube containing living luminous bacteria in blood-serum was compared with a set of standards from dilutions of oxygenated *Limulus* haemocyanin to represent the shade of blue color present at definite percentages of oxyhaemocyanin. Corresponding oxygen tensions for each percentage of oxyhaemocyanin were taken from the haemocyanin-oxyhaemocyanin dissociation curve of Redfield, Coolidge, and Hurd.[27] Color standards were made as shown in Table I, using ten uniform test-tubes.

The killed bacterial suspension was added to give to the color standards the same degree of turbidity that is exhibited by an experimental tube prepared according to the same method.

A series of indicator tubes prepared as above served for comparison with a standing tube of *Limulus* oxyhaemocyanin containing 1 cc. of *living* luminous bacteria whose oxygen consumption was to be measured. The exact time was

taken whenever the experimental tube compared exactly in color with one of the standards. The blood-serum was thoroughly shaken with air until completely in equilibrium; the time was taken from the moment the luminous bacteria were added to the tube of serum. As the oxyhaemocyanin was reduced by the bacteria in the experimental tube confusing yellowish pigments appeared with the diminution of the blue color of the oxyhaemocyanin. A small amount of Orange G added to the color standards compensated for this color change in the experimental tube.

In Table II the corresponding oxygen tensions and volume percentages of oxygen for each standard tube are given. It will be seen that no color change occurs until the bacteria have reduced the oxygen concentration in the experimental tube to a value corresponding to 28.50 mm. Hg tension (3.75 per cent oxygen). This will correspond with standard Tube 8, one being scarcely able to distinguish a color change between standard No. 8 and standard No. 9, while no visible change from blue to less blue occurs above this value of oxygen. Oxyhaemocyanin is within 10 per cent of complete equilibrium with the air at this value of oxygen, and no distinction in color can be seen between 90 per cent and 100 per cent oxyhaemocyanin.

Considerable time was therefore required for the bacteria to consume enough oxygen to cause a reduction of the oxyhaemocyanin to a point resulting in color changes at which determinations could begin. From a beginning value of 23.50 mm. oxygen to a point equivalent to standard No. 1, or 3.75 mm. (0.493 per cent oxygen), it was possible to follow the changes in the color of the experimental tube and to obtain a curve for color values against time. The oxygen consumption curve obtained by this method is limited only by the extent of the color changes of haemocyanin when passing from the oxygenated to the reduced condition. Fig. 1 shows this relation plotted against time.

At the point of lowest possible concentration of oxygen estimated by the colorimetric method, the suspension of luminous bacteria in the serum was found to be still aglow. Dimming does not occur until after a complete reduction of the oxyhaemocyanin has been brought about. This may be determined by observing the experimental tube in the dark beside a control tube kept shaken and in equilibrium with the air. When the dimming of the experimental tube begins, it is easily noted by comparison with the brilliancy of glow in the control.

Table II gives complete data from a single experiment of oxygen consumption determination.

In this experiment no dimming of the suspension of luminous bacteria was detected until a total time of 16 minutes had elapsed, a point well beyond the time at which visible reduction of the oxyhaemocyanin had occurred. Hence, it is not possible to follow the rate of oxygen consumption beyond the point of dimming by this method. The character of the oxygen consumption curve beyond this point has been investigated by the manometric method to be described later in the present paper.

TABLE II

Experiment 2, July 11, 1927

Time in minutes and seconds	Standard tube No.	Per cent OxyHcy.	Oxygen tension in mm.	Per cent oxygen in suspension	Log. of oxygen tensions
0.00	10	100	—	—	—
1.00	10	100 (?)	—	—	—
2.00	9	90	34.50	4.53	1.538
2.45	8	80	28.50	3.75	1.455
3.10	7	70	23.25	3.27	1.366
3.40	6	60	18.25	2.40	1.264
4.25	5	50	13.75	1.81	1.138
5.30	4	40	10.50	1.38	1.021
6.15	3	30	7.60	1.00	0.881
7.30	2	20	5.25	0.690	0.760
10.00	1	10	3.75	0.493	0.574

It may be noted in Fig. 1 that as oxygen consumption proceeds, equal parts of oxygen are consumed in equal times, independently of oxygen concentration (a "zero-order" reaction), down to a very low value of approximately 20 mm. Hg, before a marked reduction in the rate of oxygen consumption occurs. This is below a point corresponding with standard Tube 7, equivalent to 23.25 mm. oxygen, and standard Tube 6, which corresponds to 18.25 mm. oxygen. This is quite unlike the determinations made for larger animals where the oxygen consumption is proportional to the pressure over long periods of time and wide ranges of pressures. We cannot conclude that oxygen consumption of luminous bacteria is proportional to the oxygen

concentration except possibly at very low values of oxygen below the point of dimming. This is observed also in the complete curve obtained by manometric methods. Dimming of the suspension of bacteria occurs after the decrease in respiratory rate, indicating that the point of dimming is not the exact point at which oxygen concentration becomes just inadequate for maximum respiration of the cell.

The decrease in the rate of oxygen consumption must occur at a point where the active reactant oxygen is diminished to a value not

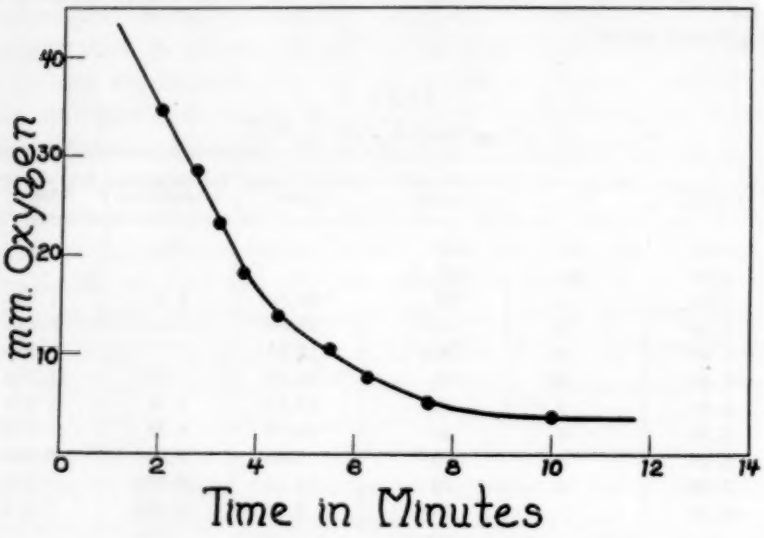

FIG. 1. Oxygen consumption of a suspension of luminous bacteria

quite sufficient to completely activate the respiratory mechanism of the cell, assuming that oxygen diffuses readily into these small organisms. This method for the determination of oxygen consumption is added to these studies to check later observations, and to call attention to the use of a colorimetric method for detection of the rate of oxygen consumption by small organisms. It should be borne in mind that there is no removal of the reaction product, carbon dioxide, but since *Limulus* blood is very well buffered, no inhibitory effects on the activity of the bacteria occurred, and the hydrogen-ion concentration of the serum did not change during the course of an experiment. The

greatest disadvantage of this method is that the range of the indicator is extremely limited and judgment of color values may often be inaccurate.

Colorimetric Determination of Carbon Dioxide Production by Luminous Bacteria

The amount of carbon dioxide produced in a standing tube containing a suspension of luminous bacteria in sea-water has also been determined by a colorimetric method. Haas[9] and Saunders[28] have previously used indicator dyes for the determination of carbon dioxide production by both marine and fresh-water animals. Henderson and Cohn[12] have published a table for the relation of the pH of sea-water to its carbon dioxide tension. By the use of indicator dyes it is possible to follow changes in the pH of a bacterial suspension in sea-water as the bacteria produce carbon dioxide and effect the bicarbonate buffer equilibrium.

Allowance was made for the salt-error introduced, from the tables of Kolthoff and Furman,[16] and careful check was made on the changing pH of the suspension in time. Curves plotted for the pH were expressed in terms of carbon dioxide tension according to the data of Henderson and Cohn. Suspensions of the bacteria were always shaken into complete equilibrium with the air and added to a test-tube containing the proper amount of the indicator (Phenol red), while a like amount of the suspension was added to a test-tube without dye to serve as a control for observation in the dark to establish time for dimming. At the end of each observation, when the experimental tube had reached the lowest pH measureable by the indicator, air was bubbled through the suspension to drive off the carbon dioxide produced and allow the suspension to return to near its original pH. This indicated that the change in pH was almost entirely due to the presence of the carbon dioxide produced by the bacteria. If the indicator did not return to within 0.15 of the original pH, the experiment was disregarded.

Although luminous bacteria are very active producers of acid, as Hill[14] has shown, a great acid production occurs only when they are suspended in a medium containing carbohydrates. Since these experiments were always conducted with bacteria suspended in a non-nutrient medium free of carbohydrates, the acid production was very slight, the only source of acid being from the carbohydrates of the cells themselves and dead bacteria in the suspensions. The experiments were conducted over relatively short time intervals, and acid production at no time became too great for reading the pH values or such as to obscure results. It will be seen from Fig. 2 that in a series of experiments on carbon dioxide production in samples from a single suspension of luminous bacteria, there is a slight

rise in the hydrogen-ion concentration of the suspension at all times. This is the only evidence of the slight amount of non-volatile acid that is produced by the bacteria as the suspension becomes older. However, only in old suspensions and suspensions made from old cultures were there sufficient quantities of acid to cause error to determinations. Consequently, all of the experiments were conducted with fresh and young, brilliantly-glowing cultures.

In Fig. 2 the rise in the amount of carbon dioxide produced in the suspension proceeds at a constant rate from equilibrium with the air

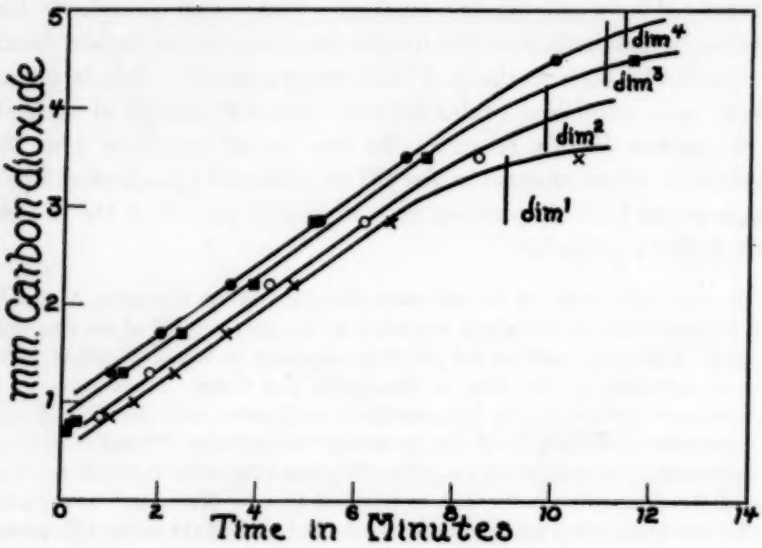

FIG. 2. Carbon dioxide production in a suspension of luminous bacteria. (Four determinations.)

to near the point of dimming, as one should expect if luminous bacteria consume oxygen independently of the concentration down to a low value of oxygen pressure. Before the point of dimming a slowing of the rate of carbon dioxide production occurs, comparable to the decrease of oxygen consumption following the lowering of oxygen to a point permitting incomplete activity of the respiratory mechanism of the cell.

In Fig. 2 a progressive increase in the time required for the dimming of a suspension of bacteria is indicated. Bacteria present in the

suspension gradually die and reduce the number of active cells. This will in turn decrease slightly the rate of oxygen consumption, allowing a longer time to be required for the reduction of the oxygen content to a point permitting dimming of the suspension because of lack of oxygen. An actual decay of the luminesence also occurs, even when the cells are adequately aerated. Hence, samples from the same suspension when repeatedly used for determinations will show a progressive

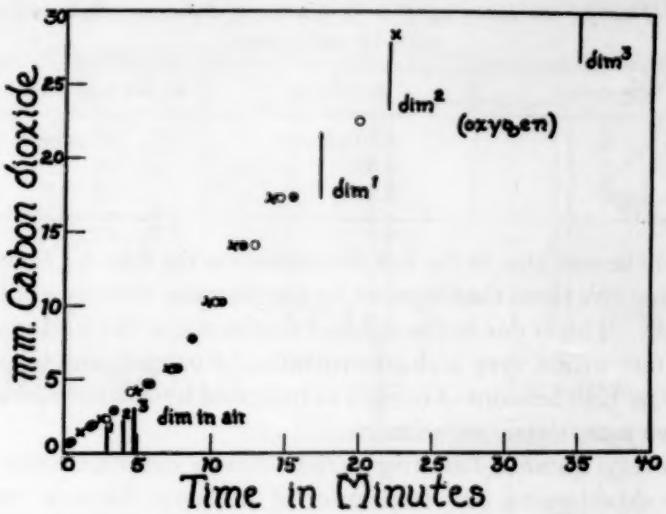

FIG. 3. Carbon dioxide production by luminous bacteria saturated with air and pure oxygen. (Three determinations.)

increase in the time for dimming. In very old suspensions a dim glow will be maintained for a remarkably long time.

Samples for carbon dioxide determinations were taken from a stock suspension that was at all times kept in equilibrium with the air and free of accumulating carbon dioxide by a stream of air continually passing through. If this stream of air is replaced by pure oxygen, it is possible to obtain a complete saturation of the suspension with pure oxygen instead of air. This was done in some cases, bringing the suspension into equilibrium with five times the amount of oxygen present in air. A five-fold increase in the time required for the dimming of the suspension would then be expected if the bacteria consume

oxygen at a constant rate independent of the pressure down to a low value of oxygen. Carbon dioxide should be produced in large quantities if all the oxygen in the suspension is consumed. This is approximately what does occur, as illustrated in Fig. 3.

Stephenson and Whetham[29b] have measured the carbon dioxide production of *Bacillus coli communis* when in equilibrium with air and oxygen and have obtained similar results.

Relative Time for the Dimming of a Suspension of Luminous Bacteria Saturated with Air and Oxygen

Determination	No. XII (air)	No. XII (saturated with oxygen)
1	3.10 minutes	17 minutes
2	4.05 "	22 "
3	5.00 "	35 "

It will be seen that in the last determination the time for dimming is *more* than five times that required for the dimming when in equilibrium with air. This is due to the reduced respiration of the bacteria when in contact with a very high concentration of oxygen, and to possible injury by high tensions of oxygen as indicated by Adams[1] and also by my own manometric experiments.

In every instance following carbon dioxide determinations in the above experiments, the suspension of luminous bacteria returned practically to its original pH on aeration.

The Effect of Oxygen Tension upon Oxygen Consumption as Determined by Manometric Methods

In the experiments of Callow[6] respiration of bacterial suspensions was successfully measured by manometric methods. In the present experiments the Thunberg-Winterstein microrespirometer was used in the form designed by Fenn,[7a] with additional modifications to permit the passage of a gas mixture into the respiratory chamber, directly above the suspension of bacteria.

By the use of a system of flow-meters, a mixture of pure nitrogen with air can be made in such a fashion that it is possible to obtain practically any desired oxygen concentration in the total mixture of gas. Pure nitrogen was obtained by passing the commercial gas over hot copper in an electric furnace. The gas was

conducted to the flow-meter through a tight system of glass and lead tubing, sealed at all joints with DeKhotinsky cement. Air was led from a second flow-meter and the gases mixed at a stop-cock connecting the two flow-meters and carried through a system of lead tubing to the water-bath containing the micro-respirometer. A complete diagram of the system is shown in Fig. 4. There was no leakage of gas or possible diffusion of oxygen into the system at any point. The only rubber connection was made very thick and extended only 2 mm. between the lead tubing and the glass tubing of the respirometer. The flow-meters were carefully calibrated for air and pure nitrogen, adjustments in pressures of the manometers giving very definite rates of flow through the capillaries of the flow-

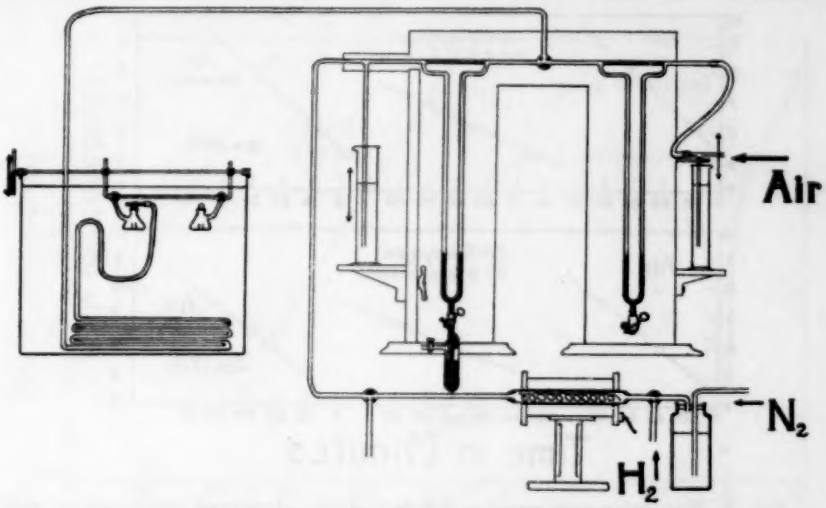

FIG. 4. Diagram of complete apparatus for determination of oxygen consumption with various gas mixtures containing partial pressures of oxygen, together with an arrangement for purification of gases.

meters. In the flow-meter conducting pure nitrogen, even the fluid of the manometer was thoroughly shaken with the pure gas before each experiment to drive off dissolved oxygen. At the microrespirometer the gas was brought into equilibrium with the bacterial suspension by a thorough shaking of the entire instrument with the mixture of gas passing through.

It was possible to make quick changes from one gas mixture to another and satisfactory determinations of oxygen consumption were easily made by this method. In each case a preliminary measurement of the rate of oxygen consumption of the particular suspension of bacteria was made in air before the suspension was subjected to a gas mixture containing only a low partial pressure of oxygen. After determinations of the rate of oxygen consumption in one or two gas

mixtures, the suspension was again brought into equilibrium with air and a check determination made to indicate if there had been a loss of respiratory activity due to decrease in the number of active cells during the course of the experiment. In each case a return to the original rate in air was made by the experimental suspension of the organisms. The suspensions were greatly diluted by phosphate buffer solution of pH 7.0, and brought into equilibrium with air by thorough shaking before a sample (1 cc.) was introduced into the respiratory chamber of the microrespirometer. The whole apparatus was rocked mechanically in the water-bath, the temperature change varying no more than 0.2 of a degree during 8 hours. The speed of rocking made no difference in the rate of movement of the indicator

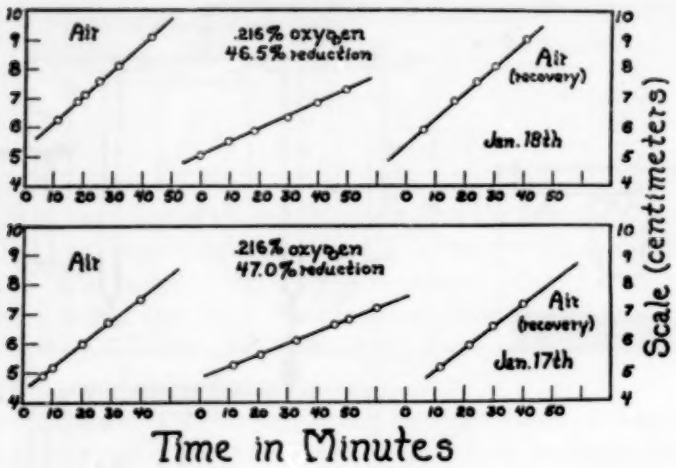

FIG. 5. Two microrespirometer determinations of oxygen consumption with equal partial pressures of oxygen.

drop of kerosene in the capillary, so long as enough movement was maintained to keep the bacterial suspension in equilibrium with the gas mixture as determinations were being made.

When gas mixtures containing the same partial pressure of oxygen were brought into equilibrium with different suspensions of bacteria, respiring at different rates in the respirometer, the percentage reduction of the respiration was nearly identical for the same gas mixtures, although the total amount of oxygen consumed by the two suspensions differed widely. This occurred in every case. An example of two determinations with different suspensions of bacteria, but identical gas mixtures is given in Fig. 5. The partial pressure of oxygen in the

gas mixtures was 1.64 mm. Hg (0.216 per cent). The reduction of respiration in the two cases is in almost exact agreement. Equally close determinations with other suspensions and other gas mixtures were made, although the agreement was less marked when only very minute traces of oxygen were present.

The value of 0.26 per cent by volume or 1.97 mm. Hg partial pressure of oxygen has been given as the point at which luminesence of luminous bacteria just begins to dim. This is in agreement with observations made during respiration in the present experiments.

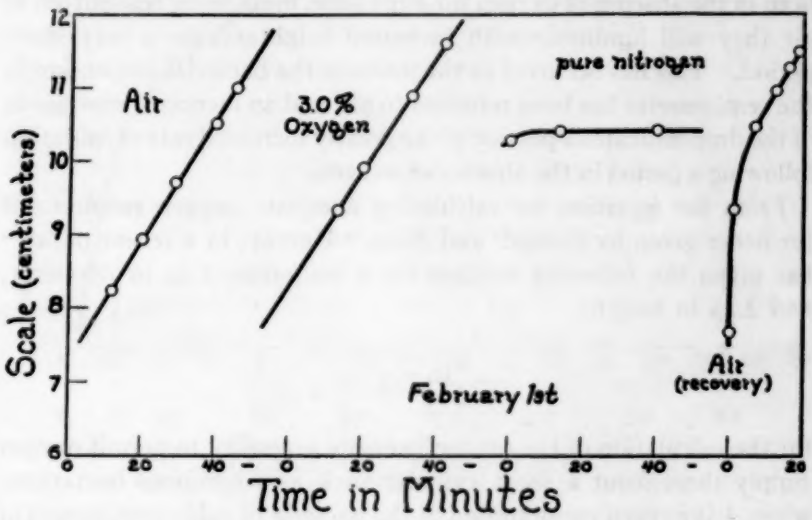

FIG. 6. Effect of pure nitrogen on oxygen consumption by luminous bacteria as measured in the microrespirometer.

When a gas mixture containing a partial pressure of oxygen equivalent to that in air (152 mm.) is brought into equilibrium with a bacterial suspension in the respirometer, there will be no increase in the rate of oxygen consumption over that at 22.80 mm. oxygen. If, however, the bacterial suspension is brought into equilibrium with pure oxygen, the rate of oxygen consumption will greatly diminish and no recovery to the original rate occurs when the suspension is again returned to air. The cells are irreparably injured by very high pressures of oxygen. This is in general agreement with the results

obtained in experiments on carbon dioxide production in equilibrium with pure oxygen.

When the bacterial suspension is brought into equilibrium with pure nitrogen, the respiratory activity quickly ceases, and no recovery occurs until the suspension is again brought into contact with air, whereupon an oxygen debt is indicated as the drop in the capillary of the instrument will move rapidly across the scale, finally falling in rate of movement to that previously obtained in air.

It has been indicated previously that after luminous bacteria have been in the absence of oxygen for some time, upon being readmitted to air they will luminesce with increased brightness for a very short period. This has occurred at the moment the bacterial suspension in the respirometer has been returned to air, and an increased movement of the drop indicates a portion of the greatly increased rate of oxidation following a period in the absence of oxygen.

From the equation for calculating adequate oxygen requirement for nerve given by Gerard[8] and Fenn,[7a] Harvey, in a recent paper[11c] has given the following relation for a bacterium 1.1μ in diameter, and 2.2μ in length:

$$C_o = \frac{Ar^2}{5D}$$

for the calculation of the oxygen pressure necessary to permit oxygen supply throughout a short cylinder such as a luminous bacterium, when A is oxygen consumption of the bacteria in cubic centimeters of oxygen per gram of bacteria per minute, r is the radius of the cylinder, and D is the diffusion coefficient for oxygen for the bacteria in cubic centimeters of oxygen diffusing per square centimeter with a pressure gradient of 1 atmosphere per centimeter, the assumption being made that oxygen consumption is independent of oxygen concentration at every partial pressure of oxygen. The calculated value for C_o obtained from Harvey's (1928) previous measurements of respiration comes out 1.53×10^{-5} atmosphere* of oxygen at the surface of the bacterium to maintain adequate respiration. This theoretical value

* Dr. Harvey informs me that this value, 1.53×10^{-5} atmosphere, should not have been corrected for solubility of oxygen in sea-water as was done in his paper.

is indeed far from the actual value, for as indicated in the curve for per cent of respiration in Fig. 7, the *observed* limiting value for adequate respiration is near 0.03 atmosphere, or 22.80 mm. Hg oxygen. All pressures above this value to equilibrium with air, permit a constant and maximum rate of oxygen consumption and at every partial pressure below this value there occurs a decrease in respiratory rate. Oxygen consumption is not independent of oxygen pressure over the

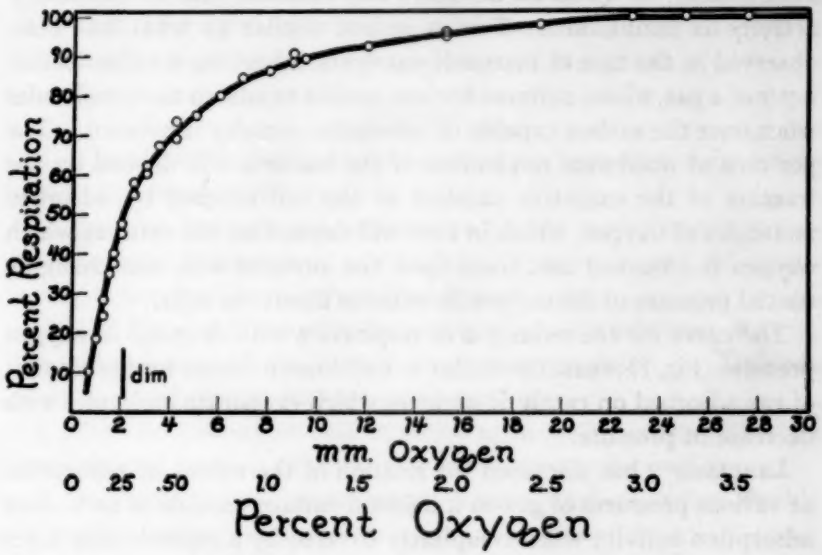

FIG. 7. The effect of oxygen tension upon oxygen consumption as measured by manometric methods.

whole range of respiratory activity, and the formula does not apply to luminous bacteria because of this assumption in the equation that oxygen consumption is always independent of oxygen pressure. The luminous bacteria are so small that the oxygen collecting at the catalytic surface of the oxidation mechanism of the cell becomes the limiting factor determining the rate of oxygen consumption rather than the oxygen diffusing into the cell.

When the partial pressure of oxygen in the gas mixtures in equilibrium with the suspension of bacteria are lowered from a value for adequate respiration, the decrease in respiratory activity does not

diminish in proportion to oxygen pressure over the whole range of oxygen concentrations. Oxygen consumption becomes about proportional to oxygen pressure only at low concentrations of oxygen following the dimming of a suspension of bacteria, when the respiration rate is reduced one-half. The curve for the reduction of respiration suggests adsorption of the gas in solution at a catalytic surface, reaching a saturation at approximately 22.80 mm. oxygen (0.03 atmosphere), at which an adequate and constant rate of respiratory activity is maintained. This is indeed similar to what has been observed in the case of inorganic catalysts, adsorbing a unimolecular layer of a gas, whose surfaces become unable to adsorb more molecules when once the surface capable of adsorption activity is covered. The per cent of maximum respiration of the bacteria will depend on the fraction of the oxidative catalyst of the cell covered by adsorbed molecules of oxygen, which in turn will depend on the rates at which oxygen is adsorbed and freed from the surfaces with the changing partial pressure of the oxygen in solution about the cells.

The curve for the reduction of respiration with decrease in oxygen pressure (Fig. 7) is exactly similar to well-known curves for the amount of gas adsorbed on catalytic surfaces which evaporate molecules with decrease in pressure.

Langmuir[18a] has discussed the relation of the extent of adsorption at various pressures of gas on a uniform surface capable of no further adsorption activity when completely covered by a unimolecular layer of adsorbed molecules, but which frees molecules of gas from the surface with decrease of pressure in agreement with an equilibrium:

$$k_1 p\,(1 - \theta) = k_2 \theta$$

when θ is the fraction of adsorbing surface covered, $(1 - \theta)$ the fraction of surface bare of adsorbed molecules, p the gas pressure, and k_1 and k_2 velocity constants characteristic of rates of condensation and evaporation from the surface respectively. If θ is considered as per cent of respiration in the curve of Fig. 7, and $(1 - \theta)$ as per cent reduction of respiration, the same relations hold, and θ will be nearly proportional to the pressure only at low pressures of oxygen, and practically independent of the pressure near saturation of the oxidative catalyst of the cell, at which point the reaction becomes one of "zero-order,"

perfectly independent of increasing gas pressures. At this high pressure of oxygen near saturation, $(1 - \theta)$ will be of small value, but will vary inversely as the pressure. The curve in Fig. 7 is a typical adsorption type, and is of the same form as curves from the data of Langmuir for adsorption of oxygen and nitrogen at mica and glass surfaces, and agrees with the form of those given by Pease[25] for adsorption of a number of gases at copper surfaces. I regard the data obtained for oxygen consumption at various pressures as supporting the view that the respiratory catalysts in the luminous bacteria are acting in a manner similar to those used in inorganic oxidations.

SUMMARY

1. The respiration of luminous bacteria has been studied by colorimetric and manometric methods.

2. *Limulus* oxyhaemocyanin has been used as a colorimetric indicator of oxygen consumption and indicator dyes were used for colorimetric determination of carbon dioxide production.

3. The Thunberg-Winterstein microrespirometer has been used for the measurement of the rate of oxygen consumption by luminous bacteria at different partial pressures of oxygen.

4. The effect of oxygen concentration upon oxygen consumption has been followed from equilibrium with air to low pressures of oxygen.

5. Luminous bacteria consume oxygen and produce carbon dioxide independent of oxygen pressures from equilibrium with air (152 mm.) to approximately 22.80 mm. oxygen or 0.03 atmosphere.

6. Dimming of a suspension of luminous bacteria occurs when oxygen tension is lowered to approximately 2 mm. Hg (0.0026 atmosphere) and when the rate of respiration becomes diminished one-half.

7. Pure nitrogen stops respiratory activity and pure oxygen irreversibly inhibits oxygen consumption.

8. The curve for rate of oxygen consumption with oxygen concentration is similar to curves for adsorption of gasses at catalytic surfaces, and agrees with the Langmuir equation for the expression of the amount of gas adsorbed in unimolecular layer at catalytic surfaces with gas pressure.

9. A constant and maximum rate of oxygen consumption occurs in

small cells when oxygen concentration becomes sufficient to entirely saturate the surface of the oxidative catalyst of the cell.

I wish to express my great debt to Prof. E. N. Harvey who first suggested this problem, for his advice, criticism, and encouragement, and also to Dr. R. N. Pease, of the Department of Chemistry, Princeton University, for some information regarding adsorption phenomena.

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NOTE ON THE NATURE OF THE CURRENT OF INJURY IN TISSUES

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Previous workers have found the current of injury uniformly negative but experiments on *Nitella*[1] indicate that it can be made either positive or negative according to the method of treatment. It seemed desirable to inquire whether this divergence could be explained, in part at least, by the fact that we employed single cells while previous workers have investigated tissues.

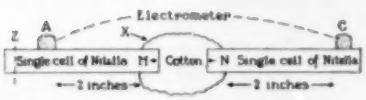

FIG. 1.

FIG. 1. Diagram to show the arrangement of an experiment. Two cells from different plants are placed in contact with a piece of absorbent cotton, *X*, and we then lead off from *A* and *C*. The letters *M* and *N* designate the ends of the cells. One cell is cut at *Z*.

By way of introduction let us consider an experiment[2] arranged as in Fig. 1 which shows two cells of *Nitella* taken from two separate plants and placed in contact with a piece of absorbent cotton. If we place 0.001 M KCl at *A*, and *C*, the cotton being wet with the same solution, we find on cutting the left-hand cell at *Z* that the potential difference of *A* (recorded with reference to *C*) becomes much more negative (Fig. 2), then quickly becomes more positive, after which it gradually approaches zero. This behavior is like that of single cells as described in former papers (in which case the experiment was arranged as shown in Fig. 3).

[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673.

[2] The material and technique were as described in previous papers unless otherwise stated. *Cf.* (1) and *J. Gen. Physiol.*, 1927–28, **11**, 391. The experiments were carried out at room temperature averaging about 22° or 23°C.

There is no essential difference between these two cases, because, although the circuit in Fig. 3 passes through A and C and that in Fig. 1 includes[3] A, M, N, and C, the electromotive forces at N and C usually cancel out since they are opposite and almost equal, and only A and M are altered and they may be regarded as corresponding to A and C in Fig. 1.

The situation is different when the experiment is arranged as in Fig. 4. Here we have two cells in their natural union, which consists of a cell wall (W), as shown in Fig. 5; this is only a few microns in

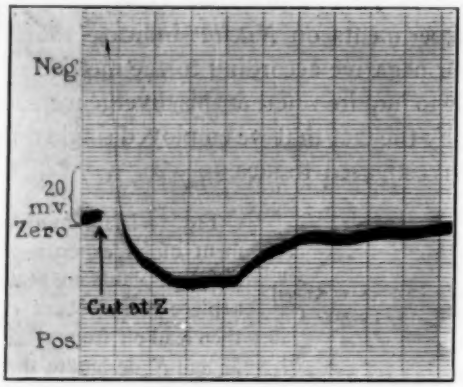

FIG. 2. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.001 M KCl at A, C, and X. When the left cell is cut at Z the curve (which records the state of A with reference to C) shows that A becomes more negative, then more positive, and that the electromotive force then approaches zero. The vertical lines represent 5-second intervals. Selected as typical from 10 experiments.

thickness and in this case is imbibed with tap water. If the left-hand cell is injured so that sap[4] comes out at M, diffuses through the cell

[3] M and N represent the protoplasmic layers at the ends of the cells.

[4] The sap is equivalent in these experiments to 0.05 M KCl. *Cf.* (1). A good method of observing the coming out of sap at M is to arrange an experiment as in Fig. 4 with an additional contact at a spot, B, a little to the right of N. We put 0.001 M KCl at A and B and sap or artificial sap at C. On cutting at Z the A to C curve becomes negative and then positive after which it slowly rises to zero as sap comes in contact with N but the B to C curve does not change unless sap diffuses along to B.

CORRECTIONS

On pages 48 to 54, Vol. 13, No. 1, September 20, 1929, through a mistake in printing the image of the string in Figs. 2, 6, 7, 9, 12, and 14 appears double. To obviate any misunderstanding it should be stated that in the original photograph the string was always single, as in Fig. 13.

wall,[5] and comes in contact with N it is clear that there is a greater difference between N and C (which is in contact with a cell wall imbibed with 0.001 M KCl). When all the E.M.F. has disappeared from the protoplasm of the cell at the left[6] (as the result of cutting at Z) the positive current will tend to flow from C (in contact with 0.001 M KCl) through the electrometer and the cell at the left (which now acts merely as a conductor) to N which is in contact with sap or a dilute sap (which acts like a solution of KCl more concentrated than 0.001 M). This

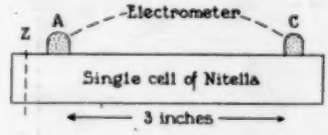

FIG. 3.

FIG. 3. Diagram to show the arrangement of an experiment. We lead off from A and C. The cell is cut at Z.

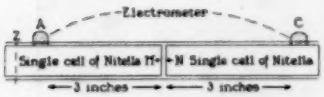

FIG. 4.

FIG. 4. Diagram to show the arrangement of an experiment. Two cells are employed, their natural union being left intact. We lead off from A and C and cut at Z. The letters M and N designate the ends of the cells.

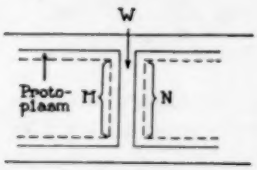

FIG. 5.

FIG. 5. Enlarged view of the point of union of the two cells shown in Fig. 4. The letter W designates the cell wall: inside this is a delicate layer of protoplasm surrounding the sap of the large central vacuole. The letters M and N designate the ends of the cells (as in Fig. 4).

gives a negative current of injury which is wholly due to the uninjured cell and which may last a long time (*i.e.*, until the cell at the right begins to lose its E.M.F. as the result of injury). Possibly this is the sort of negative current of injury observed in some cases by workers who employ groups of small cells. It is quite different from the negative current of injury in a single cell of *Nitella* which usually

[5] The cell wall is very permeable.

[6] There is some P.D. due to the cell wall which would in the present case tend to make A appear somewhat more negative than it actually is. *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761.

lasts only a few seconds (*cf.* Fig. 2) after which the cell dies and the protoplasm[6] soon loses its E.M.F.

We assume that if sap did not come out at M[7] we should get a curve like that in Fig. 2, *i.e.*, the changes in the circuit would be confined to A and M: but if sap exudes at M and comes in contact with N the curve will tend to reach a fixed negative value, the time

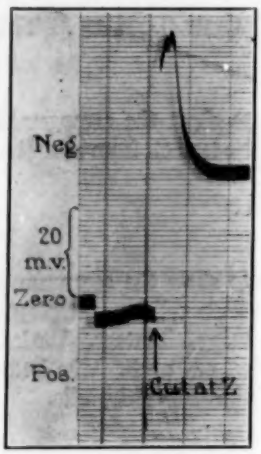

FIG. 6.

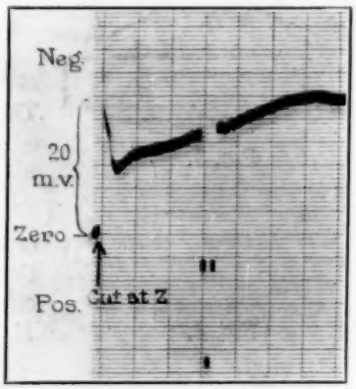

FIG. 7.

FIG. 6. Photographic record of potential differences, the experiment being arranged as in Fig. 4 with 0.001 M KCl at A and C. When the left cell is cut at Z the curve (which records the state of A with reference to C) shows that A becomes more negative after which the E.M.F. falls to a fixed value which is regarded as due to the coming out of sap at M (Fig. 5) which affects N, making it more negative (*cf.* Fig. 8*a*). The vertical lines represent 5-second intervals. Selected as typical from 40 experiments.

FIG. 7. Like Fig. 6 but showing a different result which is regarded as due to the slower exit of sap (*cf.* Fig. 8*b*). Selected as typical from 39 experiments. (Test for reversibility at about 16 seconds.)

depending on the speed with which the sap diffuses across the cell wall from M to N. That this time is variable is evident from Figs. 6 and 7.

[7] At the ends of the cells (*i.e.*, near M and N) there are a few very small cells exterior to the large cells forming the axis of the plant but owing to their small size it is not believed that they affect the observed P.D.

If the cell wall is imbibed with 0.001 M KCl the situation may be represented diagrammatically as in Figs. 8 *a* and *b*, where the curve[8] labelled *A* to *M* represents the P.D. of *A* with reference to *M*, and the P.D. of *N* with reference to *C* is represented by the curve labelled *N* to *C*. The observed P.D. between *A* and *C* (labelled *A* to *C*) may be regarded as the sum of these two curves and may take a variety of forms.

If before performing the experiment we apply 0.05 M KCl at the joint so that the cell wall between *M* and *N* becomes imbibed with it we may get such a curve as that shown in Fig. 9 and the situation may

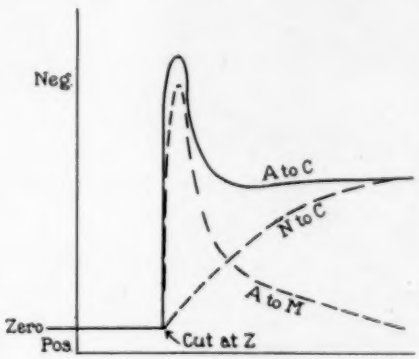

FIG. 8 *a*.

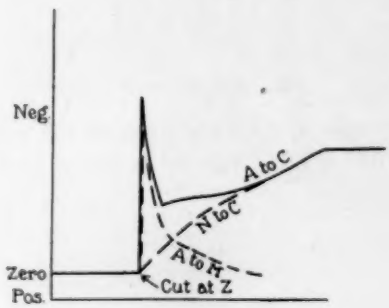

FIG. 8 *b*.

FIG. 8, *a* and *b*. Hypothetical diagram of the changes in P.D. following a cut (at *Z*, Fig. 4) when the cell wall is imbibed with 0.001 M KCl and this solution is also applied at *A* and *C*. The P.D. of *A* with reference to *M* (*cf.* Fig. 4) is represented by the curve "*A* to *M*;" that of *N* with reference to *C* by the curve "*N* to *C*." The observed P.D. ("*A* to *C*") is the sum of these two curves. Figs. 8 *a* and 8 *b* represent two forms of such curves (*cf.* Figs. 6 and 7).

be represented as in Fig. 10. The *A* to *M* curve is positive at the start and the *N* to *C* curve is negative: here too it is found that the curve may take various forms, one of which is indicated in the diagram.

In order to see how far this applies when a larger group of cells is

[8] The curves *A* to *M* in Figs. 8*a* and 8*b* are taken from actual curves obtained in cutting single cells (arranged as in Fig. 3) with 0.001 M KCl at *A* and sap or 0.05 M or 0.1 M KCl at *C*. (*Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen Physiol.*, 1928–29, **12**, 355.) In some cases such curves after passing through a negative phase become positive before reaching final equilibrium at zero.

involved experiments were made in the manner shown in Fig. 11. Bundles of plants were employed, the ends of the bundle being allowed

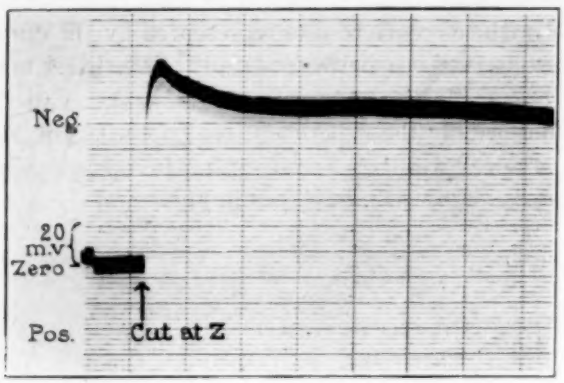

FIG. 9. As in Fig. 6 but the cell wall which separates the cells is imbibed with 0.05 M KCl. Selected as typical from 15 experiments.

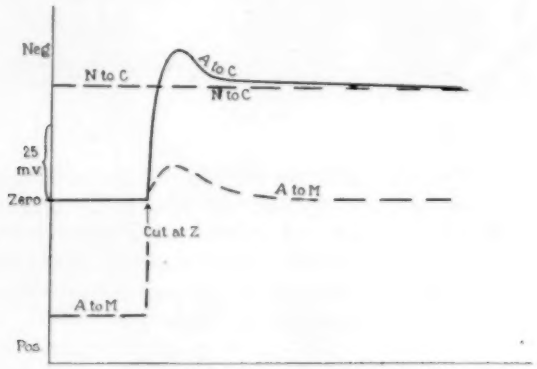

FIG. 10. Hypothetical diagram of the changes in P.D. following a cut (at *Z*, Fig. 4) when the wall separating the two cells is imbibed with 0.05 M KCl and 0.001 M KCl is applied at *A* and *C*. The P.D. of *A* with reference to *M* (*cf.* Fig. 4) is represented by the curve "*A* to *M*," that of *N* with reference to *C* by the curve "*N* to *C*." The observed P.D. "*A* to *C*" is the sum of these two curves. (*Cf.* Fig. 9.)

to dip into two dishes, *A* and *C*, in which were calomel electrodes (the cut was made at *Z*). With *A* and *C* filled with 0.001 M KCl we obtain

curves similar to that in Fig. 12 and the negative current of injury persists for many minutes.

It would seem that if our conception of the process is correct it should enable us to predict what will happen with other concentrations, for example, using the arrangement shown in Fig. 1 with 0.1 M KCl at *A* and *C* and with the cotton at *X* soaked with 0.1 M KCl, we should expect a curve like that obtained with a single cell,[9] since

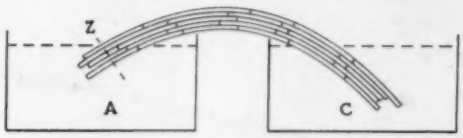

FIG. 11. Diagram to show the arrangement of an experiment in which a bundle of plants was used, the ends of the bundle dipping into the vessels *A* and *C*. The bundle was cut at *Z*.

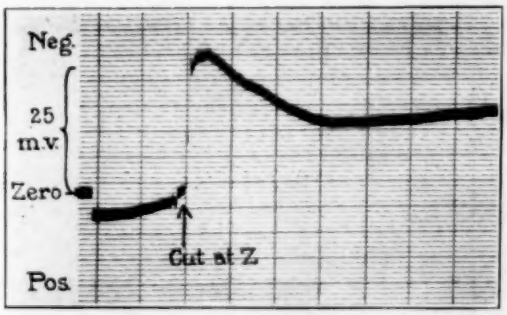

FIG. 12. Photographic record of P.D., the experiment being arranged as in Fig. 11 with *A* and *C* filled with 0.001 M KCl. The vertical lines represent 5-second intervals. Selected as typical from 5 experiments.

the coming out of sap would not affect the result and at the end we should be leading off at two points, *N* and *C*, both in contact with 0.1 M KCl. This is the case as is shown by Fig. 13.

Using the arrangement shown in Fig. 4 we should expect a similar curve at the start but later on, after the E.M.F. of the cell at the left

[9] Even with single cells there is a good deal of variation in the time required for the curve to rise to zero.

has disappeared (as the result of cutting at *Z*) and sap comes out of *M* into the cell wall (which is imbibed with tap water) and reaches *N* the positive current will tend to flow through the electrometer from *N* (now in contact with sap or dilute sap) to *C* (in contact with 0.1 M KCl) and as the curve records the P.D. of *N* with respect to *C*

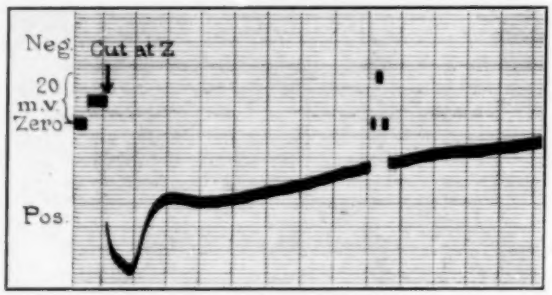

FIG. 13. As in Fig. 2 but with 0.1 M KCl at *A* and *C*. Selected as typical from 10 experiments. Test for reversibility at about 45 seconds.

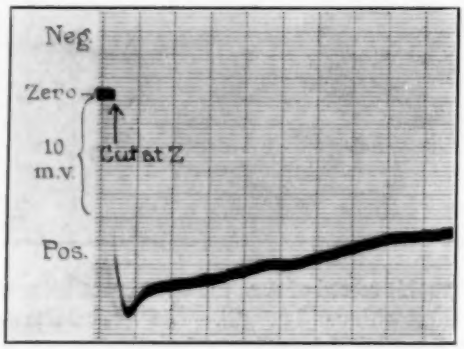

FIG. 14. As in Fig. 6 but with 0.1 M KCl at *A* and *C* and with the wall between *M* and *N* imbibed with tap water. Selected as typical from 45 experiments.

we shall expect the curve to remain positive. The more slowly the sap comes out of M the more slowly the curve will rise toward zero (this varies greatly) but even when the end wall becomes completely imbibed with sap we shall expect the curve to remain somewhat positive since sap in these experiments is equivalent to 0.05 M KCl.

That these expectations are realized is evident from Fig. 14. In this case the presence of a second cell tends to prolong the positive phase of the current of injury. The result is much the same when we employ the arrangement shown in Fig. 11.

It is evident that whenever the fluid bathing the exterior of the cell is less "negativating" than the fluid contained in the cell (as is the case in *Nitella* when 0.001 M KCl is applied to the exterior) and we lead off from the injured cell to intact cells the action of the latter will tend to prolong in a marked degree the negative phase of the current of injury.

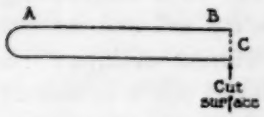

FIG. 15. Diagram of a muscle cut at one end: leading off from *A* to *C* gives more P.D. than leading off from *B* to *C*.

FIG. 15.

When we employ chloroform the injury spreads much more slowly along the cell and in many cases no spread can be detected for some minutes. In consequence the current of injury in a single cell is more lasting. This more nearly resembles the effect of cutting on muscle and nerve.[10] That there is a slow spread in muscle of the injury due to cutting is indicated by the fact that if we lead off from *A* to *C* (Fig. 15) we obtain a greater P.D. than in leading off from *B* to *C* but as time goes on this difference becomes less.

We may sum up by saying that whenever we can lead off from two places on the same cell (*Nitella*) or two places on a bundle of elongated cells (muscle and nerve) we may ascertain the current of injury due to the death process in the protoplasm.[11] But when we are not able

[10] The fibers of muscle and nerve differ from *Nitella* in that injury due to cutting does not spread rapidly and hence the current of injury lasts much longer. In *Nitella* there are two protoplasmic surfaces to consider. The experiments of one of us indicate the possibility that this may be true of muscle and nerve.

[11] This has been described for *Nitella* in previous papers. *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen Physiol.*, 1927–28, **11**, 673; 1928–29, **12**, 167, 355. See also Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920; Bayliss, W., Principles of general physiology, London, 1924, 4th edition; Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 1926, 6th edition.

to do this (on account of the small size of the cells) a lasting current of injury may be partly or completely due to the escape of sap which comes in contact with cells[12] which are intact or not sufficiently injured to lose their protoplasmic E.M.F. In plants the cell wall may also play a part.

SUMMARY

Leading off from two places on the same cell (of *Nitella*) with 0.001 M KCl we observe that a cut produces only a temporary negative current of injury.

If we lead off with 0.001 M KCl from any cell to a neighboring cell we find that when sap comes out from the cut cell and reaches the neighboring intact cell a lasting negative "current of injury" is produced. This depends on the fact that the intact cell is in contact with sap at one point and with 0.001 M KCl at the other (this applies also to tissues composed of small cells).

If we employ 0.1 M KCl in place of 0.001 M the current of injury with a single cell is positive (and is more lasting when a neighboring cell is present).

Divergent results obtained with tissues and single cells may be due in part to these factors.

[12] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761.

ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. I

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I

The upward geotropic progression of young rats has been formulated in two ways as a function of the inclination of the surface on which creeping takes place. The conditions necessary for the securing of significant measurements have been described, and need not be repeated.[1] The two formulations obtained are, first, an approximate descriptive relation between the mean angle of upward orientation (θ) and the slope of the surface (α), such that θ is, to a degree adequate for some purposes, a rectilinear function of *log sin* α; and second, in a manner consistent with the assumption that the limiting condition for steady creeping is given by sensible equality of stretching tensions on the legs of the two sides of the body during progression, *cos* θ decreases rectilinearly as *sin* α increases. When genetically comparable individuals are employed, the constants in these equations are repeatedly recoverable from litters of the same inbred strain. Different inbred strains have been available, moreover, for which these equations are again applicable, but with different numerical values of the respective constants.

Geotropic animals in which the mechanics of the support of the weight of the body on an inclined plane is certainly different from that in young rodents show that the angle of oriented progression (θ) is again a function of α, but that the sort of relationship obtained changes according to the structural conditions, so that θ may be directly proportional to *sin* α, or again to α, rather than to *log sin* α, or even that $\Delta \log \theta / \Delta \alpha = const.$ (*cf.* Wolf, 1926–27; Crozier and Stier, 1928–29; 1929–30, *a;* Kropp and Crozier, 1928–29; Kropp, 1929; Hoagland,

*National Research Council Fellow.

[1] Crozier and Pincus, 1926; 1926–27, *a, b, c, d;* 1927–28; Crozier, 1928.

1929; Crozier, 1928; several other instances remain to be described). Consistent with these findings regarding empirical descriptions of the geotropic orientation, each of the instances cited provides its own more rational equation in terms of the limitation of oriented creeping through the distribution of tensions in the supporting musculature.

Such findings strengthen the opinion that the equations concerned may very well have a functional significance beyond their value as means of empirical description. "The genetic utilization of such differences, supposing them to be found, can provide upon the one hand a biological test of the reality of the behavior constants, and upon the other a means for the consideration of certain concrete aspects of behavior in relation to inheritance" (Crozier and Pincus, 1927–28). This amounts to an application of the atomistic conceptions of genetics in the search for a biological proof that differences recognized mathematically in the behavior of two otherwise closely comparable organisms may have a "real," that is structural or molecular, basis.

We are under very real obligation to Professor W. E. Castle for his kindness in supplying us with animals necessary for the experiments, and for his interest in the progress of the tests.

The observational basis for the theoretical superstructure employed in the present discussion has been developed in a series of papers by a number of workers in this Laboratory (*cf.* CITATIONS). Important features of our conclusions from certain of these data have been adversely criticised by Hunter (1927), Piéron (1928, *a;* 1928, *b*), and Hovey (1928)—the latter working under the direction of A. R. Moore. We are quite sure that these criticisms originate from misconceptions which are in large part not unfairly characterized as inexcusable; some of these we have indicated in previous papers (Crozier, 1928; Crozier and Pincus, 1928–29; Crozier and Navez, 1929). Both Hunter (1927) and Piéron (1928, *b*) make considerable parade of "statistical" treatment of their data drawn from experiments supposedly comparable with ours, but these writers, and Hovey (1928), carefully avoid reference to the fact that in every instance the measurements we have secured have been obtained in such a manner as to provide an "internal check" automatically emerging from the changes in the variability of the recorded magnitudes of the orientations (θ) as a function of the inclination of the creeping surface. This kind of control of the significance of the data comes about in a way which cannot possibly be influenced by the experimenter during the tests, and failure to appreciate its meaning can arise only from causes for which the present writers decline to accept responsibility.

Under conditions such as we have maintained, the specific mean magnitudes of θ, in homozygous races of rats, have been found to be quantitatively recoverable in repeated series of tests over long periods. Under conditions which many earlier trials had taught us to be unsuitable for any reliable tests, wide fluctuations in upward orientation are easily observed. The influence of light, for example, unquestionably present (together with other sources of trouble) in Hunter's (1927) experiments—and said by Hovey (1928) to be without significance!—is easily shown by such tests as the following (*cf.* also Keeler, 1927–28, with mice). On a creeping plane tilted at $\alpha = 20°$, certain individuals of our race *K* gave under weak red light mean $\theta = 44° \pm 1.9$, as compared with $44.5° \pm 3°$; $43.7° \pm 2°$; $46.1° \pm 1.6$, obtained in longer series of tests at intervals during a period of two years (the first two series are cited in Crozier and Pincus, 1926–27*a;* 1928–29; the third series is based on only twenty-two observations). As is usual at such low inclinations, creeping is slow (Pincus, 1926–27) and there is much "random movement." It is necessary to wait for the intermittent periods of active progression, in which alone is orientation evident. But when a white light of moderate intensity (approx. 1 f.c. illumination) is turned on, above and somewhat in front of the surface, creeping is for some time much faster and the random movements less in evidence. At first, θ may be almost 90°. In successive runs it slowly decreases. At the end of half an hour or more, exposure to the light being continuous, θ then becomes as low as 45°. In duplicate experiments, the mean θ obtained from such tests may be as high as 80° at $\alpha = 20°$. If the source of light be somewhat to one side of the animal, it swerves in the opposite direction, and θ may be as low as 0°; again, with exposures continuing for some time, θ slowly increases. If under dim red light a young rat be proceeding in a well oriented, straight path and a white light of even low intensity be then turned on above it and slightly to the rear, θ is sharply increased (after a measurable latent period); if the white light be then excluded, θ is seen to decrease if a brief pause or hesitation permits reorientation; in the absence of adequate lateral swerving there is no particular reason (*cf.* Crozier and Pincus, 1926–27, *a*) why the animal should change its path, since on the theory we have presented orientation in the area delimited by $+\theta$ and $-\theta$ is not geotropically constrained; this very obvious condition of the experiments seems to have been deliberately violated in Hovey's (1928) experiments, and here unquestionably accounts in part for the prevalence of high values of θ at each magnitude of α, since the rats were in this case started creeping vertically on the plane and not headed to one side. These are elementary phenomena of phototropic orientation in a situation involving competition between geotropic and phototropic vectors (Crozier and Pincus, 1926–27, *b*, *c;* Wolf and Crozier, 1927–28), coupled with the occurrence of photic adaptation. If tests are made in a room with one or more windows admitting daylight, almost any complexity of behavior may be witnessed on the inclined surface. In this connection we would emphasize the fact that the upward orientation on an inclined surface cannot possibly be regarded as due to mechanical inability of the young rat to move downward. At inclinations not too great, it is easily possible to force the animal to creep downward by appropriate

illumination, and in fact downward creeping sometimes occurs spontaneously (*cf.* Crozier and Pincus, 1928–29). We therefore reject as equally inapplicable the suggestion by Wickham (1928, p. 163) that young rodents creep on inclined surfaces because they are "afraid of falling," and that advanced by Hovey (1928), following a similar thought expressed by Hunter (1927), that only in upward progression can the animals obtain a secure footing.

The contention that the oriented upward movement of young rats upon an inclined surface is not a tropistic response because it is affected by "learning" (Hovey, 1928) is of course irrelevant even if it were true. In the paper cited no proof of learning is indeed given, because during a prolonged series of observations upon single individuals the conditions of progression change as a result of the rather rapid development of the musculature of the appendages at this period of growth (in well-nourished litters). It would be necessary to have data upon the progression of "untrained" individuals of the same effective age; it is not sufficient to compare mean angles of orientation at the beginning and at the termination of a long series of trials. Comparisons of this sort which we have made with rats and with guinea pigs show under suitable conditions no effect due to "learning." Moreover, in such experiments (Hovey, 1928) there enters the possibility of errors of a sort very clearly evidenced in experiments with ants. During an investigation of the geotropic orientation of ants of various species it was found by Dr. T. C. Barnes, and by Mr. B. F. Skinner, that when an individual of *Aphaenogaster fulva* is kept isolated from the nest and is tested at intervals during several days, the angle (θ) of oriented progression at a given slope of surface shows definite increase. But this rise in θ is also exhibited by an individual kept in isolation for a similar period without trials upon the sloping surface.

The possibility of "facilitation" effects ("learning") was very early explored in these experiments.[1] No trace of it could be found, under the conditions of our tests. But again there are misleading occurrences which require that the observer exercise reasonable intelligence. With 8 individuals, for example, tests were made at 7 values of α to determine if a second series of trials, several hours subsequent to a first series, would yield a different mean θ. It was found, using 20 observations with each individual at each slope in each series, that θ was in these 56 tests on the average 1°.32 *less* in the second series than in the first. The difference $(\theta_1 - \theta_2)$ was therefore contrary in sign to what would be expected for the effect of a valid influence of "learning." It happened that these experiments were so planned that values of θ_1 were obtained in the morning, of θ_2 in the late afternoon. While the absolute differences never exceeded $(\theta_1 - \theta_2) = -5°$ in any one case, and in the mean gave $-1°.32$, the difference is entirely consistent with the known lessened activity of the young rodent in the afternoon and evening, as contrasted with morning hours, even when external conditions have been kept constant; this has been established by Szymanski (1920, 1922), and others, and for young individuals with greater technical refinement by Dr. E. Wolf and by Dr. T. J. B. Stier in the course of investigations now in progress, in which the effects of feeding have been controlled. So far as it goes, the result of these tests merely con-

firms the indications from the application of other criteria, namely that the technic of the present experiments has been relatively free from gross sources of inadequacy. It should be added that when an additional load is carried, θ at given (low) α is increased, but that tests made immediately after the removal of the load (*cf.* last section of the present paper) show θ-values uninfluenced in any way by the preceding experience.

The considerations adduced by Piéron (1928, *a; b*) are of a somewhat different order. Arguing, reasonably enough, that if the limitation of upward orientation in such a gasteropod as *Agriolimax* (Wolf, 1926–27) is controlled by the adjustment of gravitationally induced tensions in the musculature of the body as we have considered in the case of rodents, then the amount of upward orientation should be less if the animals are put under water. However, as experiments made by one of us (W. J. C.) some years ago had shown, the slug *Limax*, as used by Piéron, is not exactly suitable for such tests. When placed on a glass surface and then lowered under water the direction of subsequent turning is largely influenced by the mode of lowering of the plate into the water, and by the relation of the aquarium to the positions of windows (although the cephalic tentacles are not fully everted). What actually happens in such trials we shall discuss in detail elsewhere. When observational errors of these kinds are excluded, and allowance is made for the fact that the foot does not adhere to glass under water, and that the slugs do not creep, there is also in Piéron's account an apparent error of curious sort regarding the measurement of the angles of orientation. For the present, we would therefore cite the facts revealed in a long series of experiments in Cuba by one of us and Dr. A. E. Navez, employing *Onchidium* and various land snails of which the foot adheres well under water, and which creep vigorously; it is found: (1) that the "angle effect" in orientation is perfectly real, in air *or* under water; (2) that by the attachment of a cork float to the shell a form such as *Liguus* can be forced to orient *downward* under water, rather than upward as in the absence of the float; (3) that the reaction-time to geotropic excitation, obtained by a method which avoids handling, is a function of α, and of the mass of loads added to the shell (in air or in water). These findings are in perfect accord with the proprioceptive origin of geotropic orientation, and flatly contradict Piéron's contention. We are therefore quite justified in dismissing Piéron's conclusions as having no weight in this matter, since his alleged phenomena are in fact superficial accidents having no bearing upon the point at issue.

II

It should be obvious, of course, that persistent self-consistency in the performance of animals within a well-inbred line must be due to likeness of "inheritance." The two profitable questions which arise have to do with (1) the genetic behavior of recognizable differences when unlike lines are crossed, and (2) the possibility of utilizing the resulting genetic criteria for testing the functional interpretation of the

original differences. This amounts to a mode of definition of an hereditary effect, or of a gene, namely in terms of the connection between its organic expression and the values of an effective and experimentally controllable variable. There is implied in the acceptance of this procedure the view that the customary description of genic differences is imperfect. It has come to be rather generally recognized that a major problem in the theory of genetics is that of the method whereby genic effects are produced. The analytical manipulation of genic differences, however, clearly requires something more than mere description in terms of developmental rates and times of onset. It in fact necessitates the characterization of genetic contrasts in terms of magnitudes having functional dimensions. In only one group of instances have data of this sort been provided, namely in the case of eye-facet number in Drosophila; and here the analysis of the relations (to temperature, in this case), has certainly been defective.

The proposition we here advance serves to bring out one aspect of a general method of biological analysis which we believe to hold promise of wide significance. In outline, this method of defining an effect in inheritance seeks to avoid the pitfalls of the pseudo-quantitative treatment which depends upon mere differences in terms of a relationship of "*greater*" or "*less*" between two values of a certain variable; it does so by attempting to obtain the law of the relation between each expression of the variable in question and some controllable external variable. Instances will be given in which (*cf.* Crozier and Pincus, 1927–28) this kind of description can be shown not merely desirable, but *necessary*.

It is not to be taken for granted that the primary variable,—in cases such as we now chiefly discuss, the difference in geotropic behavior between two groups of young rats,— is in itself likely to be a simple thing. But we may illustrate the necessity for the introduction of an additional coördinate in such analyses by considering briefly the case previously described (Crozier and Pincus, 1927–28); here, two races of *Rattus norvegicus*, *A* and *K*, are found to differ from one another, as regards geotropic orientation, in the way demonstrated by Fig. 1. If comparisons between these two races were to be made solely at a low value of α it might be said that race *A* is more responsive, more sensitive, geotropically, since θ is there greater for *A* than for *K* (although its workable threshold α is higher); but if the comparison

were made only at high values of α the conclusion would however be exactly the reverse; and in an intermediate region the two races would appear identical. Thus it may happen, in any given case, that the organic variable may be fundamentally altered, genetically, in the sense that the *form* of its quantitative relationship to some external condition of its expression is no longer the same. But unless the law of the relationship can be adequately established over a range of values of an independent variable, this sort of contrast between two types of organisms cannot be differentiated from that in which the absolute magnitudes (of response, let us say) are altered while the form of dependence upon the experimental variable is still the same. This again may be illustrated by means of Fig. 1.

Data obtained with race *B* are chosen, from among others available, to show that the absolute amount of geotropic response may be markedly different in two kinds of individuals, (*A*, *B*), under the same conditions, although the way in which the response is affected quantitatively by increasing the magnitude of the exciting condition remains the same. Race *B* rats were from the 11th brother x sister generation of a line which before this period of closest inbreeding was loosely inbred. For the test of which the results are given in Table I, 5 individuals were used. θ, and P.E.$_\theta$, depend upon sin α in the way already described for the other races; the number of readings at one inclination varied between 51 and 62, so that in Fig. 2 the value of σ_M as a percentage of the mean has been plotted to demonstrate that in this line also the decreasing *variability* of response is sensibly a straight line function of log sin α. At $\alpha = 70°$ the results with *B* were not quite so certain as with *K* or *A*, due to a small percentage of cases in which slipping was present, and it is to be noticed that the measure of the variability of θ correspondingly shows a slight but definite rise (*cf.* also, Crozier and Oxnard, 1927–28). The weights of these rats, on the 13th–14th day after birth, when used for the experiment, were 20.5 $\pm$ 0.91 gms.; later we shall have occasion to refer to this point. The lesser relative reliability of the mean θ at $\alpha = 70°$, although in no sense serious, in this case noticeably disturbs the position of the cos θ plot (Fig. 1) at this value of the slope (α), since cos θ is more sensitive at the higher angles.

The two types of organic difference here diagrammed might obviously be combined in such a way as to occur simultaneously (*cf.*

data in: Crozier and Pincus, 1927–28). Another illustration of the general principle may be taken from studies on the temperature characteristics of vital processes. If the addition of a reagent induces change in the frequency of heart-beat, for example, at constant

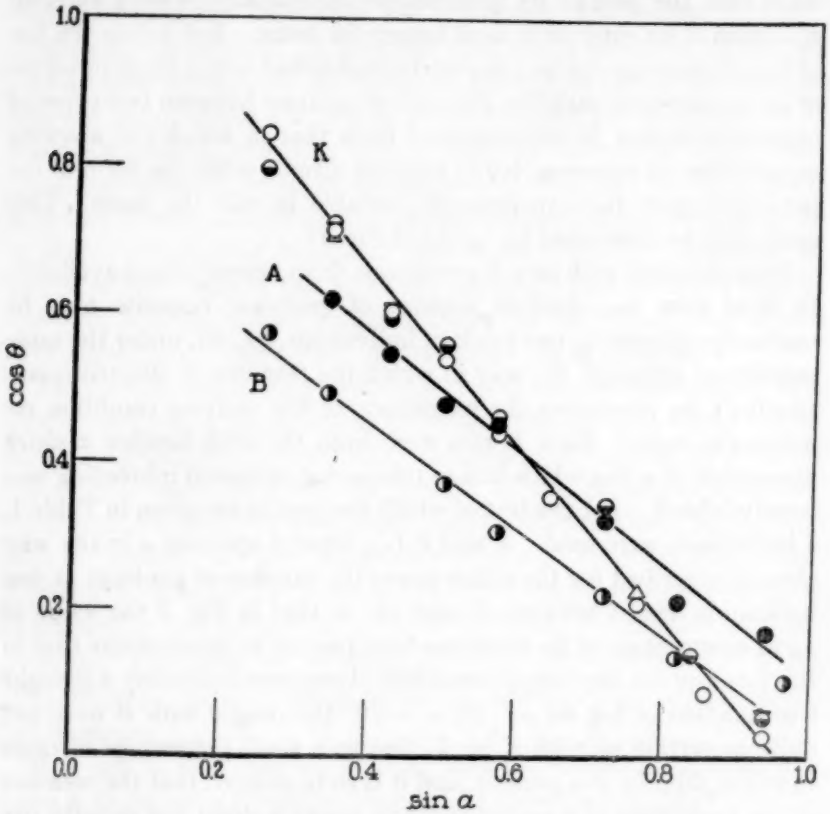

FIG. 1. Geotropic orientation of young rats of three genetically distinct races *K*, *A*, and *B*, presented in terms of the linear relationship between *cos* θ and *sin* α, where α is the slope of the surface and θ is the angle of oriented progression on the surface. (Four series of measurements are plotted for race *K*, to show reproducability; see text.)

temperature, we cannot tell from this alone whether the frequency has been altered without change of temperature characteristic, or that the whole basis of the relation to temperature has been changed (*cf.* Crozier 1925–26; Crozier and Stier, 1924–25, *a;* 1925–26, 1926–27;

1929–30, *b*, *etc.*); the mere statement that "the frequency has been increased" may be true in one part of the temperature range but not at all true in another, and by itself is of practically no analytical significance. But if the relation to temperature is identical in the presence of the reagent, though the absolute frequencies are no longer the same,

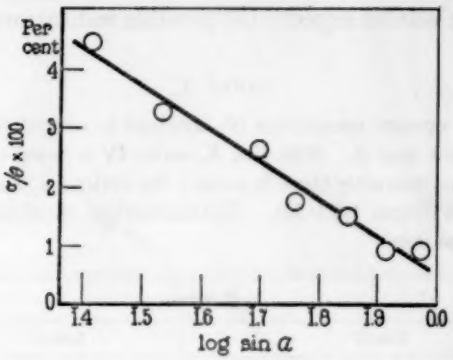

FIG. 2. Variability among measurements of θ with race *B* obeys the same rule as found with races *K* and *A;* see text.

it is clear that the mode of action of the reagent is to be described in a way which must differ materially from that required if the frequency is no longer the same kind of a function of the temperature. With regard to problems of inheritance, the point fundamentally at issue appears not unusually to be of just this kind.[2]

III

We may first consider the results of genetic tests involving races *K* and *A* in terms of the relation between *cos* θ and *sin* α (Fig. 1). On the assumption that in upward geotropic creeping the young rat orients until there is equality of stimulation by downward tensions

[2] The point we desire to make may not be completely new, but certainly it has not been expressed clearly hitherto, nor used. Thus, in discussing cases in which a particular genetic type appears different from another one only in a special environment, the best usual opinion has been (Morgan, Sturtevant, Muller, and Bridges, 1915; 1922, pp. 38 *et seq.*) that in most cases of genetic difference "the environment, being common to the two, may therefore in such cases be ignored, or rather taken for granted." In cases of the former type, it is recognized that "it is the different possibilities of reaction that are inherited." Our point is that in

on the legs of the two sides of the body, in the direction parallel to the inclined surface, it appeared[1] that it should be found that

$$\Delta \cos \theta / \Delta \sin \alpha = - const. \quad (1)$$

If the difference between lines K and A, as related to the magnitude of the slope constant in Equation (1), has an approximately simple genetic basis we should expect: (*a*) possible indications of dominance

TABLE I

Mean angles of upward orientation (θ) obtained in several series of tests with young rats of lines K and A. With line K, series IV is based upon observations (*i* and *ii*) made two years after those in series I (for series III, and the data on line A, see: Crozier and Pincus, 1927–28). The inclinations of the creeping surface are given in the first column.

α Deg.	θ, degrees					
	Line K			Line A		Line B
	I	III	IV	I	II	
15	32.6±2.66	37.4 ±				55.05±1.65
20	44.5±1.01	43.70±2.05	(i) 45.4 ±1.30	51.88±0.23	52.81±1.40	60.52±1.34
25	52.9±0.97	53.75±2.24	(ii) 52.71±1.17	56.97±0.21	58.27±1.40	
30	57.4±0.98	55.98±1.72		61.39±0.18	59.28±1.26	68.24±1.22
35	64.0±0.90	62.50±1.21		63.19±0.16	63.05±1.02	72.16±0.85
40	69.8±0.82					
45		69.89±1.18		71.24±0.14	71.44±1.05	77.26±0.78
50	77.9±0.81		(i) 75.11±1.38			
55		81.80±0.81		77.69±0.16	78.52±1.02	82.05±0.51
60	84.7±0.46					
70	88.3±0.31	86.75±0.43	(ii) 86.6 ±0.51	80.17±0.10	79.54±0.73	83.98±0.53

in F_1, but, more significantly, (*b*) evidence of segregation in back crosses between F_1 and P_K and P_A. In comparing Equation (1) with the subsequent treatment, it is necessary to remark that the deviations of the data from its curve are statistically real and significant,

either instance the "possibilities of reaction" require quantitative formulation, and that such formulation necessitates statement as a function of some independent variable. Growth-curves, survivorship curves, and dubious "reaction rate" curves do not give this information. Many years ago the investigation of the temperature coefficients for frequency of heart beat in hybrids of *Fundulus* and *Menidia*, however, was undertaken by Loeb and Ewald (1911). We expect to have more to say about such cases in the near future.

though small, even for races K and A; hence we cannot pass directly from equation (1) to the differential d θ/d log sin α subsequently used.

Line K represents about 60 generations of brother by sister matings and is identical with the King line of inbred albinos. Albinos of the

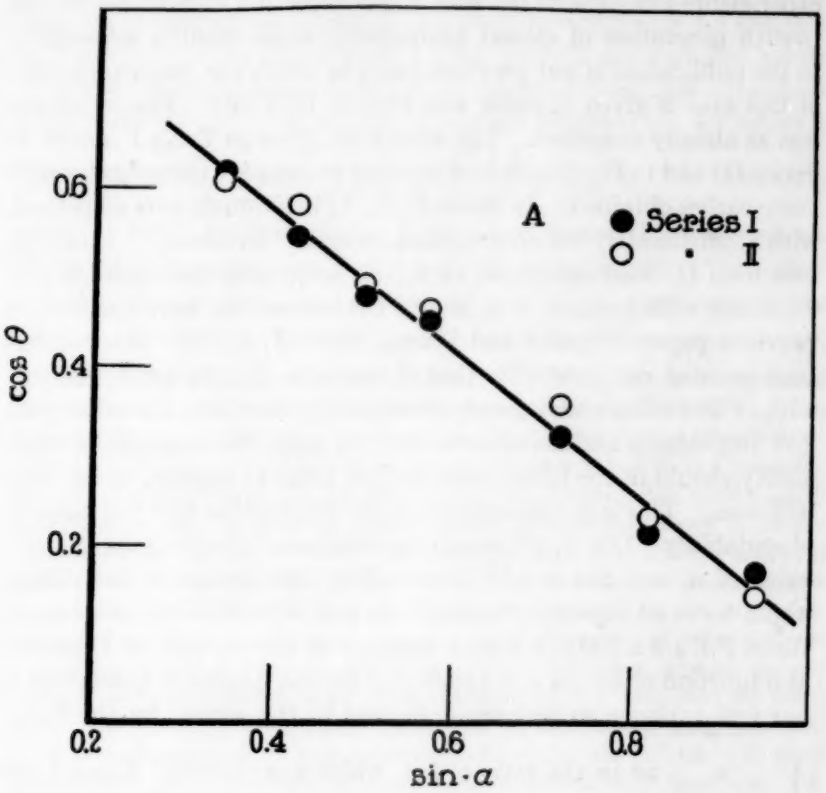

FIG. 3. Showing agreement between independent determinations of the geotropic orientation function with young rats of race A. Data in Table I (and *cf.* text).

King strain were indeed used in making the crosses for these experiments. As regards color factors, the K animals were cc RR aa hh, where c = albino, R = dark eye, a = non-agouti, and h = hooded.

The A animals were of the 10th and 12th brother x sister generation

of an inbred line of red-eyed yellow hooded animals, and as regards the color factors were CC rr aa hh. The *B* line is also red-eyed yellow hooded, and the animals tested were of the 11th brother x sister generation.

To test the reliability of the original data on the *A* line a series of experiments was undertaken with a new litter of 5 individuals (of the twelfth generation of closest inbreeding), some months subsequent to the publication of our previous paper in which the characterization of this race is given (Crozier and Pincus, 1927–28). The technique was as already described. The results are given in Table I (under *A*, Series II) and in Fig. 3. It is of interest to compare these figures with those earlier obtained. In *Series I (A)*, 12 individuals were employed, with approximately ten observations on each. In *Series II*, 5 individuals with 15 observations on each. In both cases the variability of θ declines with increase of α, and in the manner we have specified in previous papers (Crozier and Pincus, 1926–27, *a*; 1927–28). It has been pointed out (1927–28) that if two sets of tests are made, one with N individuals and *twenty* observations on each, the other with $2N$ individuals and *ten* observations on each, the measure of variability should in the former case decline twice as rapidly, as *log sin* α increases. This is in consequence of the assumption that the measure of variability $\propto 1/\sqrt{n}$, where n is the number of observations at every value of α, and also $\propto \sqrt{N}$, where N is the number of individuals which serve as separate "centers" or foci of variability of reaction. When $\text{P.E.}_\theta/\theta \times 100$ is a usable measure of the variability expressed as a function of *log sin* α, we then look for the decline of variability in our first instance to be related to that in the second by the factor $\sqrt{\frac{N}{20} \times \frac{10}{2N}}$, or in the ratio of 2:1, which was observed (Crozier and Pincus, 1927–28). This method of comparison is important, and can be generalized—roughly, it is true, but with significant results.[3]

[3] It might be objected (*cf.* Hunter, 1927; Hovey, 1928) that the frequency distributions of θ at each magnitude of α are in fact definitely skewed. To this there are several replies, not mutually exclusive. In the first place, unless number of individuals and number of observations are the same, within rather narrow limits, skewness can be introduced by differences of sensitivity (temporary or permanent) among the separate individuals (*cf.*, *e.g.*, the discussion in: Crozier and Stier,

Series I (*A*) was run in two stages, tests being made with individuals in two groups of six each, with a slight difference between the two groups. We expect that the slope of the line connecting $\text{P.E.}_\theta/\theta \times 100$ with *log sin* α to be *about* the same with the data for individuals 1–6 and 7–12. The results are given in Fig. 4. The slight age difference between the groups (1–6) and (7–12) may be responsible for the fact that the ratio of the slopes is not 1.0 but 0.83; in any case the agreement must be regarded as close. If we compare these data with those from *Series II* (*A*) (Fig. 4), we expect the slopes to be, for Series I (1–12) and Series II, in the ratio $\left(\sqrt{\frac{75}{100}}\right)\left(\sqrt{\frac{12}{5}}\right) = 1.34$. The ratio found is 1.46. Between Series I (1–6) and Series II we expect, on the same basis, a slope ratio of 1.29; that found is 1.28. We regard this sort of agreement as an excellent test of the constancy of the

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1927–28, 1928–29; Crozier and Pincus, 1927–28). Secondly, if our conception of the situation be at all correct, it is illegitimate to group the observed θ's into size classes of the same magnitude; both as regards the grouping of mean θ's as a function of the slope of the surface, and as concerns the grouping of θ's in a frequency polygon at a given value of α, the size-classes must be arranged in a series which recognizes the way in which the variability of θ is empirically found to behave (*cf.* Crozier and Pincus, 1927–28). There are two ways in which this condition can be met. One may either arrange the θ's (at given α) in a logarithmic frequency distribution, and then deal with the *geometric mean* of the θ's; or the frequency distribution may be based upon the values of *cos* θ. In either case the asymmetry evident in arithmetic seriations of observed θ's when large numbers of readings with homogeneous material are available, sensibly disappears (as is indeed obvious to mere inspection). These tests we have in fact made long ago. Since with the arrays of θ's we have used it is found that the indicated statistical refinements make no detectable difference in the usable end result, they have been ignored. But we would stress (1) the point that when for example slipping of the animals occurs, such as causes trouble in experiments with young mice at high values of α, and is easily recognized and noted, the P. E. of θ increases (both absolutely and relatively; Crozier and Oxnard, 1927–28) while the mean θ does not increase as expected; hence the two modes of treatment of the mean θ's supply important mutual checks; and (2), more significant, that the "skewness" of the frequency distributions of θ is in fact precisely what we have reason to expect if the general interpretation we have employed be essentially sound.

technic employed in the experiments, and of the homogeneity of the material constituting each set of animals tested.

It may be worth while to extend this mode of calculation to the comparison of different races as regards variability. If two races of young rats are intrinsically alike with respect to properties influencing the expression of θ as a function of α under the conditions of these experiments, then we expect the slopes of lines connecting $\text{P.E.}_\theta/\theta \times 100$ with *log sin* α to be in the ratio already given, $\sqrt{\frac{(N_1)\ (n_2)}{(n_1)\ (N_2)}}$, where N_1, N_2 are the numbers of individuals of races *1*, *2*, concerned in the tests, n_1, n_2 the respective numbers of observations at each α with

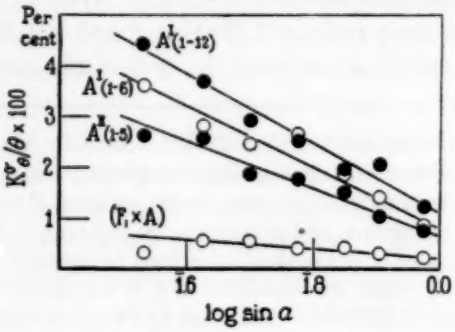

FIG. 4. Data for determining Δ (100 $K\sigma_\theta/\theta$)/ Δ log sin α for various series of observations. See Table III, and text.

each individual (constant at every α for each race). Deviation from the expected ratio would afford measure of unlikeness, as regards variability of the response, in the two races. In other words, we may compare the values of the products

$$\left(\frac{\Delta\ (100\ \text{P.E.}_\theta/M)}{\Delta\ \log\ \sin\ \alpha}\right) \times \left(\sqrt{\frac{n}{N}}\right).$$

In doing so, it has to be remembered that slight errors in fitting may make relatively large differences in the slopes, and thus in the final figures. The slopes $(\Delta\ \text{P.E.}_\theta/\theta)/(\Delta\ \log\ \sin\ \alpha)$ are obtained from plots (Fig. 4), taking $\Delta \log \sin \alpha = 0.4$. Table II contains a summary of the available data for races *A*, *K*, F_1 (*K* x *A*), *B*, and *R. rattus*. The num-

ber obtained by this procedure has the significance of a "mean change in the mean root-mean-square deviation from the mean, per individual, as a percentage of the mean, and per unit change of the independent

TABLE II

A measure of the comparative variability of the geotropic response in different lines of young rats is obtained by comparing the values of the products

$$[\Delta\ (100\ \text{P.E.}\theta/\theta)/\ \Delta \log \sin \alpha]\ [\sqrt{n/N}],$$

where α is the slope of the surface, θ is the orientation angle, and P.E.θ (or C.V.θ from weighted averages) is computed (for the *mean*) in the usual way; n = number of observations with each individual, N = number of individuals; n and N are constant, and the *same* individuals are used, at each magnitude of α in any one series. The figures in the last column are taken as direct measures of the fraction of intrinsic, internal, variability of geotropic response which is susceptible to restriction by increasing the geotropic excitation. Discussion in text. See also Table X.

(1) *Race and Series*	(2) N	(3) n	(4) $\sqrt{n/N}$	(5) $\dfrac{\Delta\ 100\ \dfrac{P.E._\theta}{\theta}}{\Delta \log \sin \alpha}$	(6) (4) x (5)
K I	2	20	3.17	1.75	5.55
K III	4	10	1.58	3.80	6.00
K II*	2	15	2.74	0.55	1.50
K + weight	4	10	1.58	1.67	2.64
A I_{1-6}	6	8.3	1.18	2.40	2.83
A I_{7-12}	6	10.0	1.29	1.80	2.32
A I_{1-12}	12	9.2	0.875	2.70	2.36
A II	5	15	1.74	1.80	3.13
F_1 (*K* x *A*)	12	10	0.915	2.10	1.92
(F_1 x *A*)	21	20	0.977	0.36	0.352
B	5	10	1.42	2.67	3.79
R. rattus	5	3	0.74	3.6	2.67

variable." Its use in this form is justified only when the individuals constituting a sample are fairly comparable with one another, but the general method has important possibilities of application to other and quite different cases. The essence of the method consists in the

formulation of *varibility as a function of some controlling variable.* Where duplicate series of observations permit direct comparisons, the agreements are clearly as close as can be expected. (It may be mentioned that for the *K* race the "variability number" can also be computed from data on the *speed* of progression (Pincus, 1926–27); it is of the order of magnitude given for angles of orientation (Table II)). And there are equally clear differences between the several groups. To interpret these differences it has to be noticed that (other things equivalent) the slope Δ (100 P.E.$_\theta$/M) / Δ log sin α is an *inverse* measure of the uncontrollable intrinsic general variability of the recorded behavior of the animals in the experimental situation, including errors of recording, but a *direct* measure of the variability of response due to factors susceptible to restriction by increasing the magnitude of the geotropic vector. In this sense, the F_1 (*K* x *A*) individuals are significantly more variable in their geotropic response than their *K* or *A* parents, so far as concerns the operation of influences (affecting the measured responses) which are susceptible to restriction by increasing the geotropic excitation; though, as we shall later be able to demonstrate, the F_1 group should, organically, be in this respect much closer to *A* than to *K*, as it is indeed found to be. We may note that at the time of observation this could not possibly have been foreseen. Although this increased uncontrollable variation in F_1 may be at first sight curious in the genetic sense, it is entirely consistent with the expected effects of hybridization, and may be related in part to the greater weights of the individuals in the hybrids than in the parents, at the time of experimentation.

When the function $\Delta\,\theta\,/\,\Delta\,\alpha$ is the same for two individuals, but θ is higher, consistently, with one than the other, this makes the P.E. of the mean θ larger than it "ought" to be; and it increases that fraction of the total variability of the mean θ's which cannot be proportionately reduced by increasing α. This phenomenon is clearly evident in our detailed records, and is very likely related to the weight of the animal. Hence our comparison with heterosis. This is still further borne out by the data from a populatiou produced in the backcross F_1 x *A* and known to be in this respect heterogeneous (Fig. 4). Again, as we shall subsequently discuss, the residual variability measured in this way and open to influence by increasing sin α, is reduced

by attaching a mass to the animal's body (*cf.* entry "*K* + weight," in Table II), as should be expected.

For completeness sake, and also because it provides an interesting check upon these contentions from a quite different aspect, we include a series of observations with rats of race *K*, Series II, already referred to (Crozier and Pincus, 1927–28) as "unsatisfactory" because of technical difficulties. The results are given in Table III, and the computed variation index is included in Table II (*K*, II*).

Direct observation led to the rejection of this series as relatively nonsignificant, because creeping was more erratic than usual, and in correlation with circumstances clear to the observers at the time (low temperature, mechanical disturbances). In spite of the fact that θ increases so much more rapidly as α increases than in

TABLE III

Orientation Data for Series II, Race K; See text

α	θ	P.E.$_\theta$ /θ x 100
degrees	*degrees*	*per cent*
15	26°.7	0.79
20	33°.5	0.81
25	41°.7	0.53
30	51°.1	0.37
35	59°.3	0.25
40	63°.2	0.27
45	66°.5	0.26
50	70°.8	0.18
55	75°.9	0.15
60	79°.3	0.16
65	80°.7	0.32

standard series with this race, Δ (P.E.$_\theta/\theta \times 100$)/ Δ log sin α is *less* than in series where no persisting sources of irregularity were apparent, and the index of variability employed in Col. 6, Table II, is accordingly much less than in the other series—proportionately less of the total variability is susceptible to geotropic control.

Individuals of the F_1 generation produced by crossing lines *K* and *A* were separately investigated, in two groups of 6 each. The F_1 progeny were of course all of the black hooded type (in view of the constitution of *K* and *A*). At the time of the tests these animals were 13–14 days old, and weighed 21.93 $\pm$ 0.20 gms.; these weights are, as was to be expected, slightly higher than in the parent stocks at the same age

(*cf.* Crozier and Pincus, 1927–28). Any gross influence due merely to increased weight would be expected (*cf.*[1] refs.) to increase θ, at a given slope of surface. But it is clear (*cf.* Table IV; Fig. 5) that the θ's actually found fall definitely within the limits set by the values provided by lines *K* and *A;* therefore, for the moment, this difference in weight can safely be ignored in the further treatment.

The results from these experiments showed that the mean θ's were strictly comparable among the separate individuals. In Table IV

TABLE IV

Mean angles of upward orientation (θ) in two groups, six individuals in each group, of young rats in the F_1 generation of crosses between lines *K* and *A*. The general means of the θ's agree closely with the averages of the means of the two groups, the former being used for subsequent computations. Since the numbers of observations at the several magnitudes of α are not the same, the variability of the measured θ's can be compared in an exact way only through the coefficients of variation of the weighted means, which are therefore given (in the last column, with their probable errors).

α	θ			C.V.$_\theta$
	(i)	(ii)	(average)	
degrees	*degrees*	*degrees*	*degrees*	*per cent*
20	46.75	52.19	49.42±1.01	3.05 ±0.42
25	53.48	55.54	54.57±0.86	2.34 ±0.32
30	62.38	59.12	60.39±1.10	2.71 ±0.39
35	64.12	58.37	62.10±0.73	1.75 ±0.24
45	68.82	69.07	68.62±0.58	1.25 ±0.17
55	77.70	75.48	76.55±0.69	1.35 ±0.19
70	82.20	81.03	81.68±0.45	0.818±0.11

the data for the two series are given, together with the general means of the average θ's for the separate individuals, with their probable errors, and the respective indices of variability. In previously recorded series of data[1] we have used experiments in which the numbers of observations were kept the same, thus permitting direct use of P.E.$_\theta$ as a measure of variability. When, as in the present series, the number of observations fluctuates somewhat (though not greatly) among the separate individuals (*viz.*, from 7 to 12) it is necessary to compute the coefficient of variation (Table IV). It was earlier demonstrated[1]

that if the number of individuals be kept *the same* at different magnitudes of α, and the number of readings made with each, the index of variability of θ then declines in a rectilinear fashion as log sin α is in-

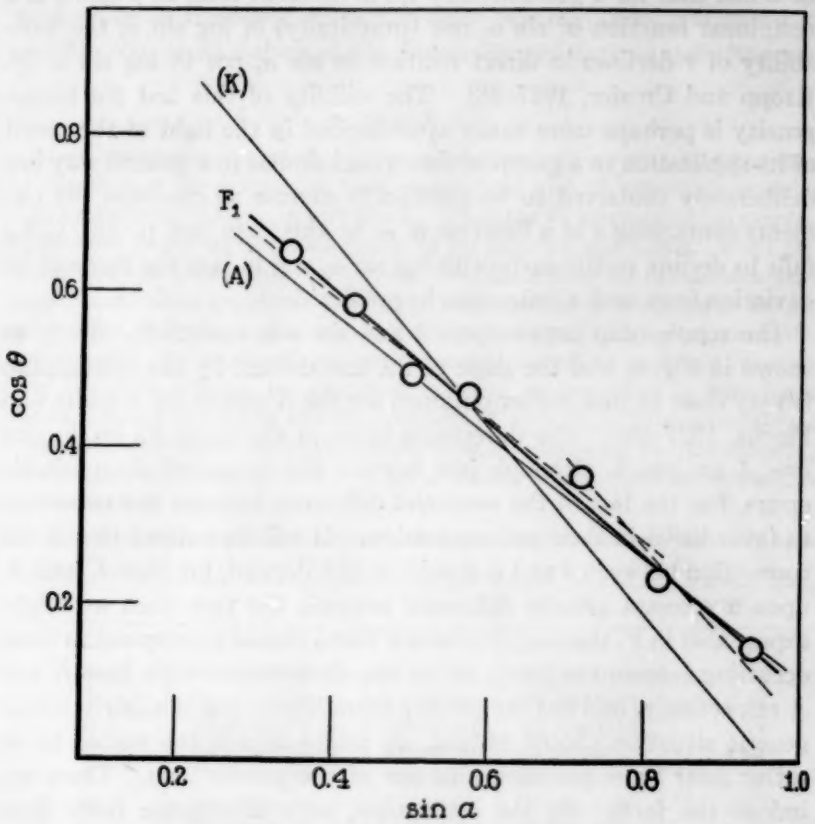

FIG. 5. Mean orientations of 12 individuals produced by crossing $K \times A$. The thin lines on the plot are those fitting the relationship $\Delta \cos \theta / \Delta \sin \alpha = -$ *const.* for the two parental races, transferred from Fig. 1. (In this figure it is assumed that the data can properly be fitted by a straight line. Another and more accurate interpretation (dashed line) is introduced subsequently.)

creased. This relationship, reasons for the existence of which are discussed elsewhere (Crozier and Pincus, 1926–27, *a;* 1927–28), becomes in fact a rather delicate test of the homogeneity of the popu-

lation of individuals employed, as well as of the conditions of the experiments themselves. In the present instance it is apparent that $C.V._{\theta}$, and its probable error, decline in the expected way. It may be noted that for a geometrically quite different case, in which θ is a rectilinear function of *sin* α, not (practically) of log sin α, the variability of θ declines in direct relation to *sin* α, not to log sin α (*cf.* Kropp and Crozier, 1927–28). The validity of this test for homogeneity is perhaps more easily apprehended in the light of the result of its application to a group of young rats similar in a general way but deliberately contrived to be genetically diverse as concerns the elements controlling θ as a function of α; in this case (see p. 78) $C.V._{\theta}$ fails to decline rectilinearly with log sin α, and in fact the mode of its deviation from such a course can be predicted.

The relationship between cos θ and sin α is essentially direct, as shown in Fig. 5, and the slope of the line defined by the relationship is very close to that earlier obtained for the *A* parent (*cf.* Crozier and Pincus, 1927–28). The differences between the mean θ's for F_1 and line *A* are small, although just beyond the ranges of the probable errors, but the fact of the *consistent* difference between the two tends to favor belief in their real separation. It will be noticed that if the connection between θ and α should in fact depend, for lines *K* and *A*, upon a relevant genetic difference between the two, then we might expect that in F_1 the magnitudes of θ found should correspond to those occurring *between* the limits set by the observations with lines *K* and *A* respectively, and not far outside these limits; and if a fairly simple genetic situation should obtain, we might expect the values to be rather near those pertaining to one of the parent lines. These are indeed the facts. At the same time, wide divergence from these expectations would not necessarily preclude genetic analysis; but we appear to have at hand a somewhat fortunate case, in which the absolute magnitudes of the orientation angles and the slope of the line connecting cos θ with sin α are both within the limits set by the parental stocks. At higher values of α, the mean θ's tend to agree rather closely with those established for line *A*, so that the gross *primary* change, in F_1, may be viewed as an alteration of the *slope* of the *cos* θ *vs.* sin α relationship.

These F_1 individuals, which should be a homogeneous group, genetically, and are shown by the statistical test to be a homogeneous group as to their geotropic behavior, were raised to maturity and crossed to lines K and A. Lines K and A had been shown through the earlier work to be in their respective ways also homogeneous, and found to provide consistent values of θ in successive generations and after con-

TABLE V

Mean orientation angles (θ) for upward geotropic progression as determined by the slope of the surface (α), from observations with 22 individuals resulting from back crosses of $F_{1\,(K \times A)}$ with line K. The mean θ's are computed as averages of the mean angles of orientation gotten for the separate individuals. If the preceding analysis is essentially correct, the individuals procured in this back cross should constitute a population genetically heterogeneous with respect to the elements determining θ as a function of α. We expect then two consequences of this heterogeneity: (1) the smooth connection between $\Delta \cos \theta / \Delta \sin \alpha$ should be noticeably disturbed, although the general character of the connection between θ and log sin α should be more or less the same; and (2) with ($F_1 \times K$) the *variability* of θ should *not* decrease in rectilinear fashion as log sin α increases, but should instead go through a minimum at about $\alpha = 35°$ or slightly above; Fig. 6 shows that this is indeed a fact.

α	($F_1 \times K$)		($F_1 \times A$)	
	θ	$C.V._\theta$	θ	$C.V._\theta$
deg.	*degrees*	*per cent*	*degrees*	*per cent*
20	52.21±0.40	1.13 ±0.11	52.54±0.99	2.85
25	57.11±0.25	0.637±0.065	57.44±0.85	2.18
30	60.82±0.26	0.638±0.065	60.83±1.14	2.78
35	66.71±0.27	0.596±0.061	64.52±0.73	1.67
45	73.50±0.39	0.786±0.080	70.14±0.27	1.71
55	78.24±0.33	0.63 ±0.064	76.00±0.99	1.93
70	80.90±0.39	0.707±0.072	80.84±0.69	1.28

siderable intervals of time (upwards of 2 years). In the back-cross generation the color and eye-factors were found to segregate in the expected ways, but we are not at the moment concerned with this. From these crosses 21 individuals were taken at random (by litters) in families $F_1 \times A$, and 22 in families $F_1 \times K$, in each case comprising an approximately equal number of males and females. These indi-

viduals were tested separately, employing standard procedure. The results will presently be considered in detail. At the moment we desire merely to consider the *average* results. If our premises are thus far sound we are prepared to encounter the following: (1) the slope of the line of mean θ's should now be consistently nearer to that of the *averages* of the corresponding values for the K and A lines; (2) the cos θ *vs.* sin α relationship, for mean θ's, might be seriously disturbed, or at least definitely modified; (3) the variability of the mean θ's

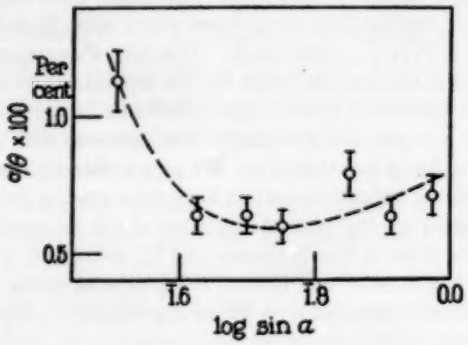

FIG. 6. The variability of θ in a population of young rats deliberately arranged to be gentically heterogeneous fails to decline regularly with increase of *log sin* α. In this case the (22) individuals derived from the backcrossing of F_1 $(K \times A)$ with K were expected to be diverse as regards factors affecting the amount of geotropic orientation; the graphs connecting θ with α for the two parental lines intersect at about $\alpha = 35°$; hence we look here for σ /M to be a minimum at about $\alpha = 35°$, which is the fact, and (*cf.* Fig. 2) we expect the descending limb of the curve to be the steeper.

$(F_1 \times K)$ should no longer be found to decline directly with increasing log sin α, but should pass through a minimum at $\alpha = 35° - 40°$, although the rising branch of the curve ($\alpha > 40°$) should not be so distinct as the descending arm ($\alpha < 40°$). These expectations arise from the fact that if segregation of factors concerned with the manifestation of θ as a function of α does indeed occur, then the mean of this $(F_1 \times K)$ population should return toward that of the two P lines, the θ -graphs for which cross at about $\alpha = 35°$ (Fig. 1).

The significant test is made by contrasting the behavior of the $F_1 \times K$ individuals with that in line K, since the F_1 data show at least *relatively* complete dominance of factors in the A line (*cf.* Fig. 5). Table V contains the necessary data. It is seen that C. V.$_\theta$ does indeed behave in the expected way (Figs. 6, 7), while it is clear (*a*) that the mean θ's are about midway toward those exhibited by rats of line K, and (*b*) that the cos θ *vs.* sin α curve does appear seriously affected.

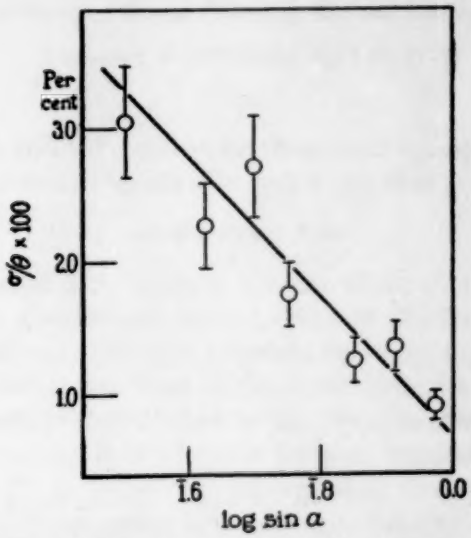

FIG. 7. Variability of θ in F_1 individuals ($K \times A$) declines with *log sin* α in an essentially rectilinear way. It may be noted that the ordinate scale is quite large; σ /M $\times$ 100 is plotted as a bar, the height being = 2 P.E. of the ordinate.

The θ-curve *slope* is more interesting than the absolute magnitudes of the θ's, as we will shortly have occasion to see. The very significant behavior of C.V.$_\theta$ we have a right to regard as a direct justification of the reasoning which initially led us to the investigation of genetically stabilized lines. Since the absolute differences which concern us are small, it is of interest to demonstrate that the rate of decline of variability of the mean θ's for the individuals from the opposite backcross, ($F_1 \times A$), is actually *less* than with the individuals from ($F_1 \times K$), as it should be if the premises already discussed are efficient. Table

V contains these data also. It is apparent that the variability declines with increasing α, as we have a right to foresee, but that there is no real minimum (C.V.$_\theta$ has been corrected for the number of observations at each slope). It is necessary to remember again that variations in weights of individuals may be significant here, or other causes leading to change of θ without change of $\Delta\,\theta\,/\,\Delta \log \sin \alpha$; these influences will affect the absolute magnitudes of C.V.$_\theta$ as computed.

ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. II

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IV

It has been pointed out that for these cases θ (mean or median) is very nearly related to *log sin* α, in such a way that

$$\Delta\theta/\Delta \log \sin \alpha = \text{"const."} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

In every series of data, however (Crozier, 1928; *cf.* Crozier and Pincus, 1927–28; Crozier and Oxnard, 1927–28; Keeler, 1927–28), the measurements really fall upon a sigmoid curve (*cf.* Fig. 8). The constant recurrence of this form of the curve forces its recognition, although we were at first content to use the approximate logarithmic relationship because it is adequate for some purposes (Crozier and Pincus, 1926–27, *b*). The two end regions of these curves are hard to establish precisely, owing to increasing variability of movement at low values of α and to difficulties of progression at very high inclinations ($> \alpha = 70°$). But there is no reason to question the general form of the curves. If we assume an array of peripheral receptors which may be stimulated in increased number (or with greater frequency) as the labor of upward progression is increased (*i.e.*, as α is made higher,) then, on the basic assumption that upward orientation ceases with sensible equivalence of excitation on the two sides of the body, the angle Θ must be taken as a direct measure of the mean total number of activated sense organs per unit of time. "Sensible equality of excitation" means that not more than one "group" of receptors is stimulated, on the average, on one side in excess of those stimulated on the other side. But the total number of activated receptors (pro-

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prioreceptive tension-receptors, on this view,) will depend not only on the magnitude of the exciting force (here proportional to *sin* α) but also on the distribution of excitation-thresholds among them. The relation of this conception to Hecht's (1923–1928) analysis of retinal excitation should be clear, although the two are by no means identical,

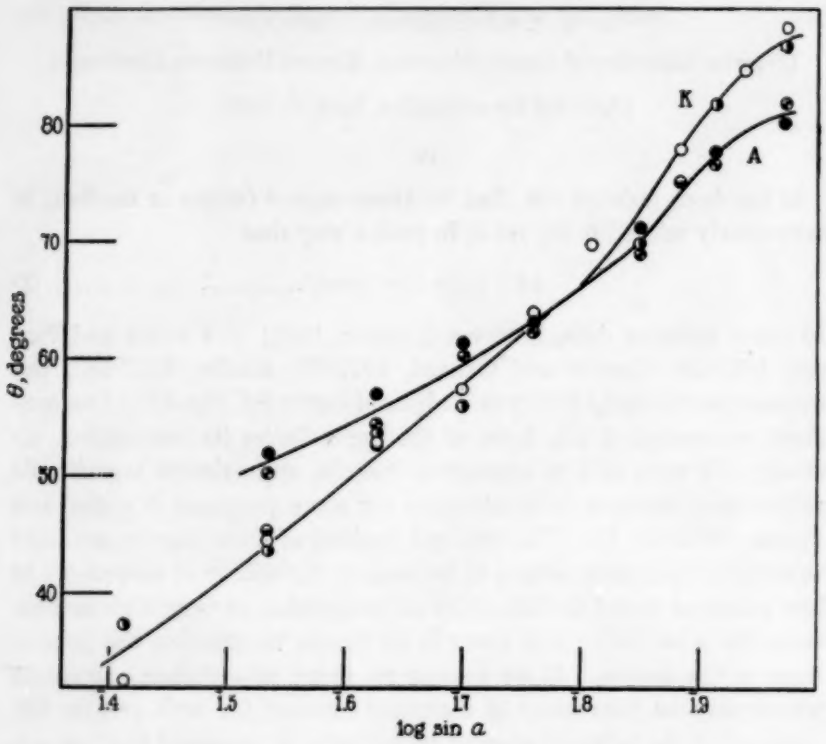

FIG. 8. θ as a function of log sin α, for lines *K* and *A*.

as we will find reason to see. The ratio d θ/d *log sin* α (that is, the slope of the curve connecting θ and *log sin* α) gives the rate at which additional excitation is involved as *sin* α increases. Unquestionably, the matter of *frequency* of excitation, as related, mainly, to the speed of creeping (*cf.* Pincus, 1926–27) and thus to the frequency of movement of the appendages, introduces complications. Tension-excitation depends on *change* of tension (*cf.* Adrian and Zottermann, 1926;

Cooper and Creed, 1927; Adrian, 1928). We are justified in exploring the consequences of simplifying assumptions, provided we do not forget their existence, and we shall accordingly assume that while the effective gravitational pull is proportional to *sin* α, the excitation of a single receptor unit over an interval of time during progression depends upon *log sin* α. It is significant here that the speed of progression, approximately determined by the frequency of stepping, is known to be directly proportional to *log sin* α (Pincus, 1926–27); more accurately, in the data at our disposal, θ and speed of progression are directly proportional.

For a given Θ, there is equivalence of numbers of tension-receptor sense organs activated on the two sides of the body, and constant *frequency* of excitation over gross intervals of time. Then θ is a direct measure of the total excitation experienced. It is on this basis that the relation $\Delta\ cos\ \theta / \Delta\ sin\ \alpha = -\ const.$ is obtained.[1] When α is varied, the speed of creeping changes, also θ. We can regard this as signifying that both the total number of receptors and the frequency of excitation alter with α, and so with θ. Hence, θ is such a function of α that, as α varies, it in some fashion includes both elements in the totality of excitation over a gross unit of time. Assuming that for any small increase, ΔE, in total excitation per unit time, we have $\Delta E = K_1 (\Delta N)(\Delta F)$, where N signifies number of receptors, F frequency of excitation through changes in the positions of the limbs during progression, then since $\Delta F \propto$ Speed of progression, at least approximately, and since Δ Speed$/\Delta\ log\ sin\ \alpha$ is sensibly constant,

$$\Delta E = K_2 (\Delta N)(\Delta\ log\ sin\ \alpha).$$

But our assumption must be that Θ and E are equivalent,

$$\therefore \Delta\theta = K_3 (\Delta N)(\Delta\ log\ sin\ \alpha)$$

and $\dfrac{\Delta\theta}{\Delta\ log\ sin\ \alpha} = K_3 (\Delta N)$. On this basis, plotting $\Delta\ \theta / \Delta\ log\ sin\ \alpha$ against *sin* α should show how increasing α brings new sense organs into play according to the distribution of effective tension-thresholds among them.

In this connection it may be remarked that lowering the temperature decreases speed of movement, and θ, at given α; while attaching (moderate) added loads increases both. It should be clear that the *logarithmic* factor in these computa-

tions is not to be considered as other than a conveniently adequate relationship; and with respect to any tendency which may exist to regard it as signifying "Weber's Law" we may point out that here the approximately logarithmic relation holds over the *whole working range*, not to its mid-portion (*cf.* Hecht, 1923–24).

The phenomena of "post contraction" in the human arm show how one aspect of the direct proportionality of continued excitation to motor effect may be tested

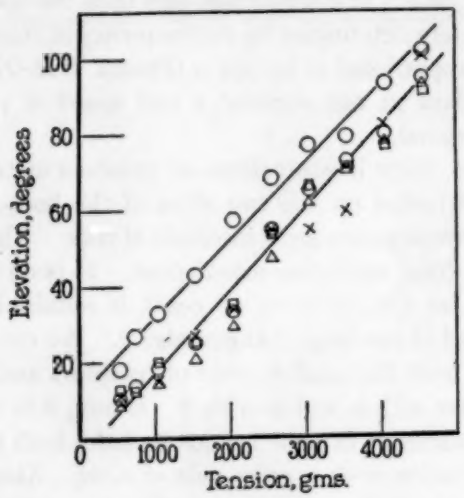

FIG. 9. Data on the angular elevation of the human arm (post contraction), released after withstanding various tensions (from: Allen and O'Donoghue, 1927). The uppermost set pertains to one subject, the lower sets to another. See text.

objectively. Allen and O'Donoghue (1927) measured the post contractional angular displacement of the arm after submission to known tensions. Although they analyze their results in a quite different way, it is clear from the data that the angular displacement, which (rather than the work done in elevating the released arm!) may be taken as a measure of the "intensity" and therefore of the *persistence* of the initially excited flow of proprioceptively originating impulses, is in fact directly proportional to the exciting force (cf. Fig. 9). In many other instances the attempt to find "logarithmic" relationships is equally out of focus.[4]

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[4] We may anticipate certain matters which it is expected to develop elsewhere, to the extent of pointing out that the nature of the limitation of geotropic orientation as here considered is essentially identical with the ancient problem of lifted weights in psychology. It can be stated that analysis of the existing data appears possible, without appeal to "units of sensation" but in terms of numbers of affected tension-receptors, by a method analogous to that employed in the present paper.

CORRECTION

On pages 85 and 86, Vol. 13, No. 1, September 20, 1929, the cuts for Figs. 10 and 11 should be interchanged. The cut on page 85 is Fig. 11 and should appear on page 86. The cut on page 86 is Fig. 10 and should appear on page 85. The legends should remain on the pages where they now appear.

It will have been noticed that for the distributions of measurements given in Figs. 8, 12, *etc.*, smooth curves have been drawn. This is entirely justified under the conditions, since each plotted point is subject to a certain (measured) precision, which is taken into account in drawing the smoothest permissible curve. It may well be that if greater refinement of observation were possible, discontinuities of an abrupt sort would become apparent, but if the notions employed in

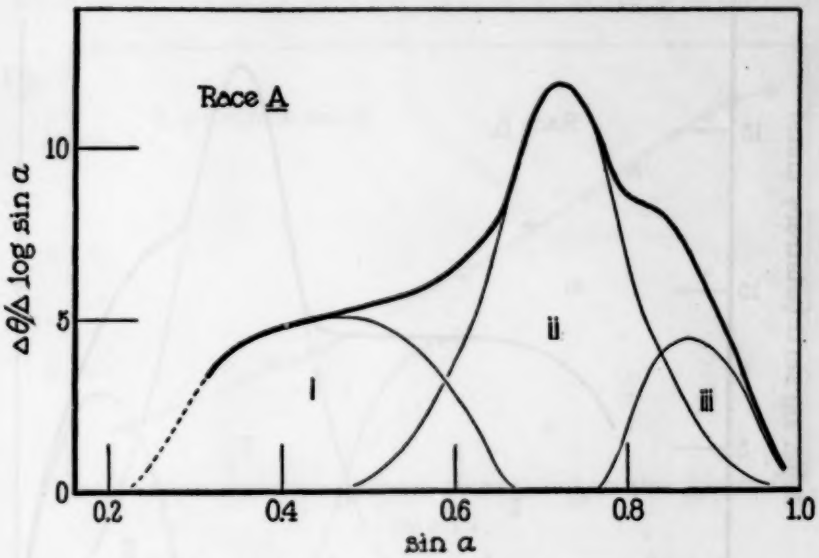

FIG. 10. In arbitrary units the differential $\Delta\theta/\Delta \log \sin \alpha$, obtained graphically, from the data for race *K* (*cf.* Crozier and Pincus, 1927–28; and Fig. 8 of the present paper) is plotted against $\sin \alpha$. The resulting curve (full line) is analyzable into a central symmetrical distribution curve, and the residues at either side provide by difference the right- and left-hand groupings. Within reasonable limits, I, II, and III are each symmetrical.

this paper are approximately correct there seems, in the case of this material, no opportunity to achieve such precision; nor is there any likelihood that it would appreciably affect the main results of the analysis even if secured.

When $\Delta\ \theta/\Delta \log \sin \alpha$, obtained by measuring the slopes of the fitted curves (Fig. 8) (by the mirror method, for example), is plotted as a function of $\sin \alpha$, the differential curves appear as in Figs. 10

and 11. These graphs clearly are not simple. But for the fact that suggestive comparisons may be made among the curves available for the several races, it might be unprofitable to attempt to use them. As they stand, the differential curves, necessarily incomplete at the low-α end, each appear to be composed of 3 almost symmetrical population curves. This can be taken to signify, if further tests support the

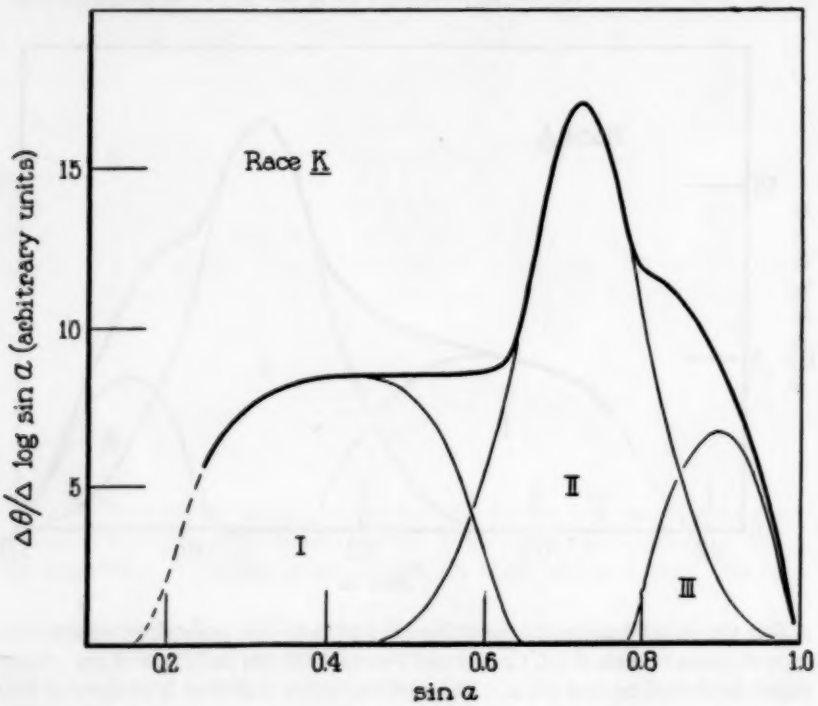

FIG. 11. An analysis of $\Delta\theta/\Delta \log \sin \alpha$ *vs* $\sin \alpha$ for race *A* shows essentially the same condition as in the case of race *K*, except that *i*, *ii*, *iii*, are each smaller than the corresponding elements in *K;* and the positions of the maxima are *slightly* different.

notion, that there are involved 3 large groups of receptors, of successively higher mean excitation thresholds, the implication of the members of these overlapping groups in the determination of the angle θ proceeding additively as $\sin \alpha$ is made larger. The three groups may be labelled I, II, III in race *K*, i, ii, iii in race *A*. Whether these "groups" are, as such, distinct entities, or that merely the mechanics

of the involvement of receptors in excitation is diversified in this way, we are not at the moment called upon to answer.

The triplicate character of these curves reappears in the analysis of the data pertaining to race *B* (Fig. 12, 13). When the three races are compared the differences between them are seen to be of the following kinds. Disregarding the magnitudes of θ at the lowest workable slopes, and paying attention only to Figs. 10, 11, 13, races *A* and *K*, which chiefly concern us, show but slight differences between the mag-

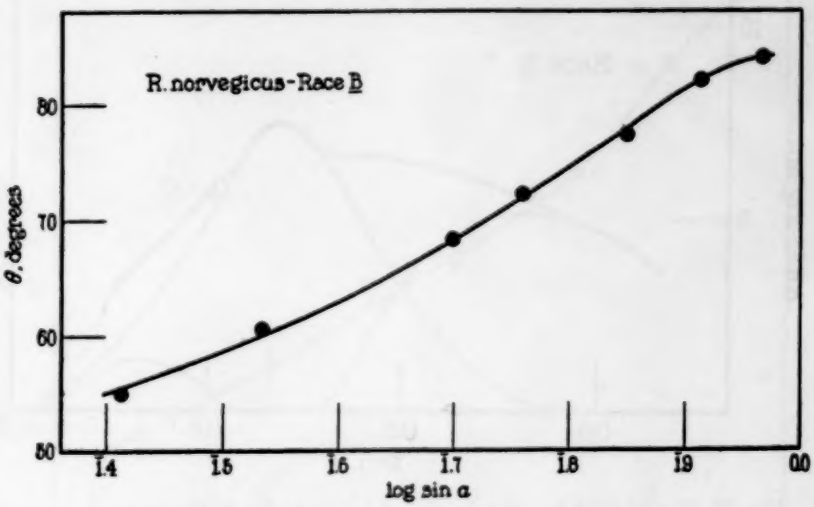

FIG. 12. θ as a function of *log sin* α, for race *B*.

nitudes of *sin* α at which the peaks of our separate distribution curves appear. The only real differences concern the sizes of the three subcurves into which each curve has been analyzed. The central curve for *A*, in fact, is drawn by multiplying the corresponding ordinates in the *K* curve by a constant. Within the limits which are completely negligible in view of the errors of the graphical procedure, the same is true of the curves for *i* and *iii*. The *A* individuals then differ from the *K* in the magnitudes of the ordinate units in which $\Delta\ \theta/\Delta\ log\ sin\ \alpha$ is to be expressed. Essential differences between *A* and *K* should be revealed by reducing the curves for the two to a common basis, involving (1) a threshold θ, (2) a constant for $\Delta\ \theta/\Delta\ log\ sin\ \alpha$. The analysis

of the θ vs. *log sin* α curve for race *B* (Fig. 13) shows groups 1, 2, 3 again easily recognizable, but to be in each case small. The similarity between *A* and *B* we have already seen to be chiefly a matter of the geotropic threshold (Fig. 1, 8), rather than of the function $\Delta\theta/\Delta\alpha$. Hence if we adjust the ordinate scale to give equivalence in rise of θ over the working range of geotropic excitation the curves for *A* and *B* should be made to coincide, at least approximately. This might be

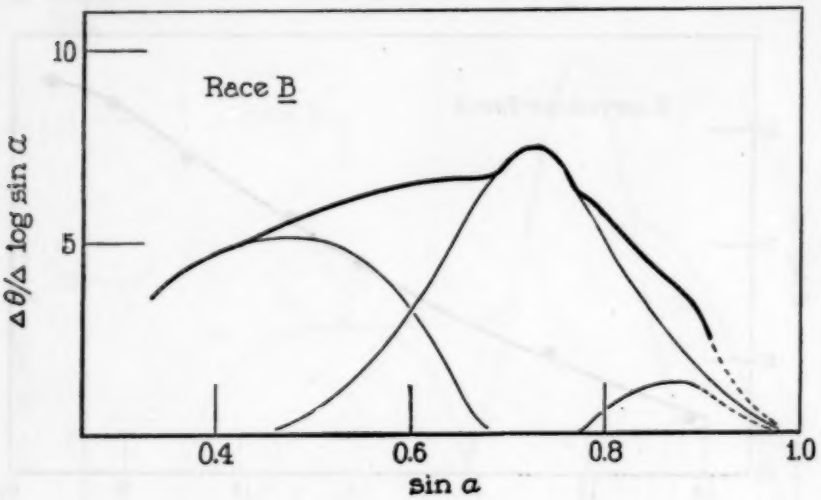

FIG. 13. Plot of $\Delta\theta/\Delta$ *log sin* α for race *B*. The modes of the 3 "groups" are at practically the same points (*sin* α) as with *K* and *A* races, but the "groups" are each smaller. (The extreme right hand end of this curve is uncertain; see text, and Fig. 2.)

regarded as the effect of a difference in a central nervous threshold determining the liminal difference in excitation on the two sides of the body. No such simple adjustment can be made with the data from *K* and *A*, however, which can abolish the differences revealed by Figs. 10 and 11.

Relations of the same sort appear in the case of young mice (*cf.* Crozier and Oxnard, 1927–28). The orientation of slugs (Wolf, 1926–27), and of other forms, involves different considerations, which cannot be gone into here.

The lowermost "group" is incompletely represented, since $\Delta\theta/\Delta$ *log sin* α cannot be shown to drop to zero within the range of values of α open to reliable test. But if we assume it to be a really symmetrical curve (*i.e.*, *I* and *i*) we can arrive at a sort of approximation of the lowest slope effective in evoking geotropic creeping. For the *K* race, this is (Fig. 10) at about 9°; for the *A*, about 5°; and for *B*, about 6°.

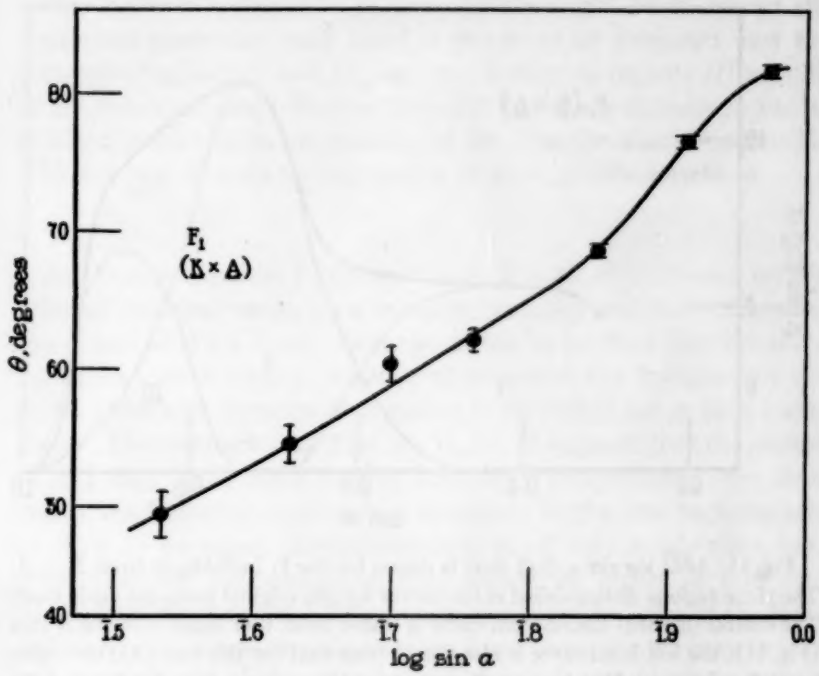

FIG. 14. θ *vs. log sin* α for the F_1 progeny ($K \times A$); points are plotted as bars with height = 2 × P. E.

These values are "ideal" however, and except in the case of *K* do not correspond to the indications of the *cos* θ *vs. sin* α plots; in this respect races *A* and *B* must be regarded as having certain of the receptors concerned in the determination of θ already activated when the individuals of the age we have employed are creeping on the flat (*cf.* Fig. 1).

We might proceed in several ways to test the adequacy of this representation. It may be possible, for example, to modify the

curves in Figs. 10, 11, 13 when the rats carry additional loads (attached masses). One might obtain in this way evidence as to the possible anatomical separateness of the three presumptive receptor groups. For the time being, however, we confine attention to a genetic test.

In Fig. 14 the connection between θ and *log sin* α is plotted for the F_1 individuals from the cross K x A. It may be mentioned that the

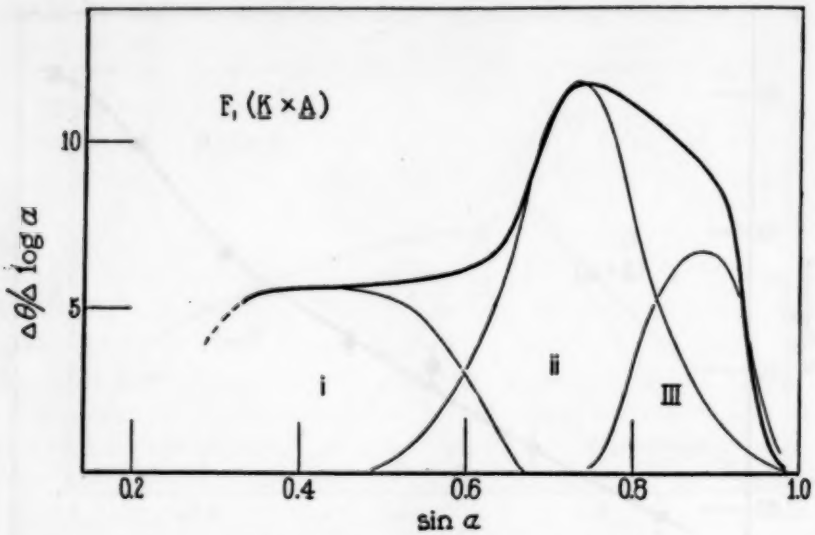

Fig. 15. $\Delta\theta/\Delta \log \sin \alpha$ (full line) is drawn for the F_1 individuals from $K \times A$. The three regions distinguished in the curves for the original races are again clear. The central (dashed) distribution curve is traced from that found with the A race (Fig. 11); the left hand curve is also traced from that for this race (A); the right-hand distribution is that for race K; except for the extreme right hand end of the graph, which is in any case uncertain, the parental resolution curves iA, iiA, $IIIK$ give an entirely adequate picture of the state of affairs in the progeny; see text.

respective curves were drawn in their final forms before this mode of treating them was contemplated. It appears (Fig. 15) that the F_1 individuals resemble the A race in that "groups" 1 and 2 are closely enough alike to be taken as identical (*i.e.*, they are *i* and *ii*), while the area and form of "group" 3 is here an exact duplication of that in race K; in fact, the constituent curves are traced from the respective graphs already given. The only differences lie in the fact that *i* in

F_1 is slightly larger than in *A*, but obviously not like *I* in *K*, so that its curve must be slightly expanded (as in Fig. 11, dashed line); and there are evident slight shifts in the positions of the maxima—that for ii being in F_1 pushed 0.06 unit to the right, and for III 0.08 unit to the left (this makes III assume, as regards its maximum, exactly the *A* position, i the *K* position, as seen in Figs. 7, 8).

The naïve interpretation of these effects might be to regard the conditions producing small *i* and *ii* groups to be dominant over the corresponding large I and II; and reciprocally as regards III and iii. If the developmental bases for these differences are distinct and independent, more critical examination of the situation should be possible with the help of tests for segregation of their genetic correlates.

V

Individuals from the F_1 generation of (*K* x *A*), already used for the orientation experiments, were raised to maturity and back-crossed to the *K* and to the *A* lines. If the suspicion be justified that the three "groups of sense organs" correspond to genetically independent entities, a different outcome is of course to be looked for in each back-cross. The comparison of Figs. 10, 11, 13, 15 suggests that the groups 1, 2, 3 may be in some fashion essentially independent. We then expect the following relationships to appear in the two back-crosses. In (F_1 x *A*) we expect the individuals to be all very nearly alike, but, given adequate measurements, they should be separable into two classes upon the basis of a difference in size of group 3; for, if the indications of dominance already seen are lived-up-to the progeny in this back-cross should obviously be of two sorts, phenotypically, one half of them (ideally) showing a large group 3, *i.e.*, *III*, the other half showing *iii*. In the other back-cross, (F_1 x *K*), we should expect the third group to be essentially alike in all the individuals, but there should be *four* classes as regards the exhibition of large and small curves for groups 1 and 2.

The kinds of difference thus predicted impose a radical test of the antecedent analysis. There is required the establishment of a reaction curve for each individual separately, the grouping of these curves upon an objective basis of the differences they may exhibit, a statistical justification of such groupings, and the examination of the differen-

tial curves which eventuate. We may expect that differences among the weights of the cross-bred individuals may introduce complications, so that this additional point must be examined experimentally.

Four litters were used in the backcross F_1 x *K*. These animals were born within two weeks of each other. Three litters were produced by mating F_1 females to 60th generation King albino males, the fourth from a mating of a 60th generation King albino female to an F_1 male. Four animals (nos. 19 to 22) were put to nurse on a *B* family female. No influence due to the mother was noticeable although the *B* line geotropic reaction is obviously different from that of lines *A*, *K* or F_1. Similarly 6 animals of one of the three F_1 x *A* litters tested were nursed by a *B* line female, with no apparent influence on the geotropic response due to the mother. The three F_1 x *A* litters were also born within two weeks of each other, and two months after the F_1 x *K* litters. They were all sired by the same *A* line male and the mothers were F_1 females. All the F_1 animals used in these crosses were previously tested for their geotropic reaction. Where the litter size was greater than six the litters were split and part put to a foster-mother. Thus from two to six animals were nursed by a single female, but never more.

The individuals tested in the back-cross (F_1 x *A*) were 21 in number, 3 litters. Each animal was tested for geotropic orientation, under standard conditions, about twenty readings being secured at each of 7 inclinations. The same creeping surface was used throughout. The mean θ's are collected in Table VI, where the individuals are grouped in a manner to be discussed shortly.

Although the numbers of rats are small, there are several independent tests which may be applied and which give concordant results concerning the reality of the differences detected in comparing these individuals. We have seen reason to expect very slight if indeed any differences in the curves at low values of α, since we would look for the receptor groups 1 and 2 (*i.e.*, *i*, and *ii*) to be identical throughout. But, from the results of experiments with attached weights[1] (*cf.* later), this expectation might be upset if the weight of the young rat is genetically modified, either as to amount or arrangement, in such a way as to act differentially upon our assumed groups of receptors. Table VII shows that there is no correlation, however, between the total

TABLE VI

Average orientation angles secured with 21 individuals (3 litters) in the back cross generation ($F_1 \times A$); separated into two groups on the basis of $\Delta\ \theta$ in the range $\alpha = 45° - 70°$; analysis in Figs. 17 and 18. In this and in several succeeding Tables blank entries signify that for one reason or another no observations were made. In the column headed "*color*", *b* signifies *black*, *y* signifies *yellow*.

($F_1 \times A$) *Group 1 (III)*

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
26	27.0	b	53.44	59.75	61.56	64.20	71.70	74.79	80.71
28	24.5	y	51.63	56.80	60.80	65.44	73.80	75.75	82.19
30	19.0	b	52.60	—	57.20	61.90	72.40	75.44	82.27
33	18.0	y	50.67	61.50	64.00	63.00	71.10	75.77	82.00
36	24.0	b	51.93	56.47	64.40	66.50	69.40	74.00	80.07
37	25.0	y	51.53	55.25	58.86	64.23	70.27	73.67	78.76
40	25.0	b	49.07	55.06	59.36	61.40	70.00	73.86	80.50
41	24.5	y	51.06	56.75	62.21	65.53	70.80	77.93	81.20
42	24.5	b	52.44	57.87	61.59	66.29	69.56	73.44	79.71
43	28.5	b	52.95	56.50	63.73	65.53	71.40	74.43	80.33
θ *mean* =			**51.63**	**57.33**	**59.37**	**64.40**	**71.04**	**74.91**	**80.77**

($F_1 \times A$) *Group 2 (iii)*

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
23	25.5	y	51.89	59.15	63.30	67.10	74.60	78.20	83.27
24	24.5	b	51.65	58.37	59.53	64.53	73.30	77.47	81.53
25	26.0	b	51.37	55.53	59.82	63.13	70.50	78.93	80.29
27	25.0	y	54.00	56.35	62.15	65.63	72.00	76.00	79.75
29	22.5	b	53.07	59.60	63.00	63.40	74.40	75.50	80.68
31	17.0	y	54.09	—	62.00	61.20	74.40	77.87	82.47
32	18.5	b	52.35	—	59.81	61.69	74.10	75.86	79.31
34	17.5	y	51.67	—	58.29	64.27	74.50	78.50	82.00
35	19.0	b	54.67	—	57.64	63.90	74.10	78.00	81.76
38	24.0	y	51.79	57.00	62.38	68.07	69.75	75.44	79.00
39	24.5	y	52.53	56.12	59.87	64.06	69.93	75.20	79.81
θ *mean* =			**52.44**	**57.44**	**61.02**	**64.31**	**72.75**	**76.90**	**80.81**

weight of individual and the mean θ at $\alpha = 20°$, nor at $\alpha = 70°$. This influence of weight we may then ignore, at least as to any *gross* manifestation (as already noted with the F_1 generation). We can

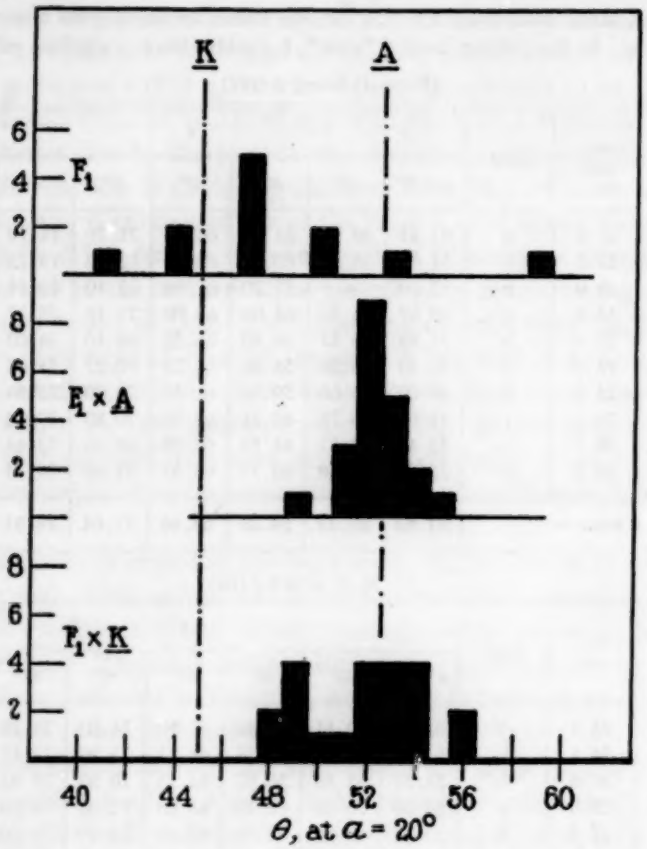

FIG. 16. Frequency distributions of mean θ at $\alpha = 20°$, for F_1, $(F_1 \times A)$, $(F_1 \times K)$ populations; mean values of $\theta_{20°}$ for K and A lines are added.

therefore afford to look for a significant shift of θ at $\alpha = 20°$ in comparing the orientations of individuals produced in the two back-crosses. It is to be remembered that at this low inclination the θ measurements are the most variable; so that we might not be surprised to find only blurred indications of the relationships sought. The experimental

facts, however, are detailed in Fig. 16. It is clear that the F_1 individuals show modal θ's between those already gotten with the *K* and *A* races, and that with (F_1 x *A*) the mode moves still further toward the *A* position—becomes in fact identical with it. With (F_1 x *K*) there is some definite indication of two types of θ, one near the F_1 position—that is, shifted toward *K*,—while the majority cluster about the θ characteristic of the *A* race. We shall find independent reason for regarding these distributions as reasonable.

TABLE VII

Distribution of mean θ for each individual at $\alpha = 20°$, as function of weight of animal; ($F_1 \times A$), and in italics ($F_1 \times K$). Showing no correlation in either case.

	Wt., gms.	17.5	19.5	21.5	23.5	25.5	27.5	29.5
θ, degrees	46.5	*1*						
	48.5		*2*	*3*	*1*	1	*1*	
	50.5	1 *1*	*3*	*1*	3	2		
	52.5	1	2 *3*	*4*		6	1 *1*	1 *1*
	54.5	1	1 *2*	*2*		1 *1*		*1*
	56.5	*1*	*1*		*1*			

The mean θ's at lower slopes for (F_1 x *A*) individuals are obviously consistent throughout. At the uppermost end of the curve, however, this is no longer true. Careful consideration of the curves for the separate individual shows that they fall into two classes which can be distinguished reasonably well. Up to $\alpha = 55°$ the θ's for both classes show astonishing agreements with the means already established for the *A* race[1] (*cf.* Table 1). The fact that the agreements are in this respect better than with the F_1 population may be accidental, or due to the genetic reshuffling of unrecognized influences affecting orientation. But above $\alpha = 45°$ the two classes of cases recognized in Table

VI are to be separated. In one of these classes the determination at $\alpha = 55°$ is consistently higher (as in the F_1 population) than in A, while the figures for the other class agree better with those for A. The curves for the mean θ's in the respective classes are given in Fig. 17. There is no real correlation between weight of individual and this grouping, though the iii rats average a little less than the III array. These differences are so slight that in spite of their complete consist-

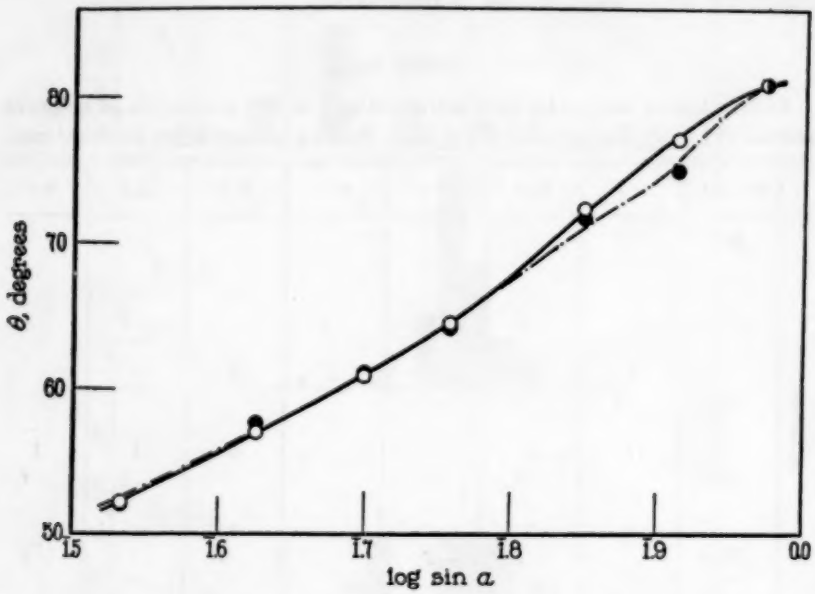

FIG. 17. Orientation-angles (θ, mean) for two groups of individuals (Table VI) distinguished in the back-cross progency ($F_1 \times A$); in one group (●), the effect labelled III (*cf.* Figs. 10, 11, 13) is apparent, and contrasts with iii in the other group; the only detectable difference is in the region $\alpha = 50°$ to $\alpha = 70°$.

ency with the forecast of the analysis we do not feel that any great significance can be attached to them alone. Their real meaning is that the mere crossing of F_1 and A individuals, found to be closely similar in geotropic behavior, does not give rise to new and unforseen differences among the progeny produced. This negative evidence is none the less powerful, and supports the evidence provided by the forms of the curves in Figs. 17, 18. The relations between *cos* θ and

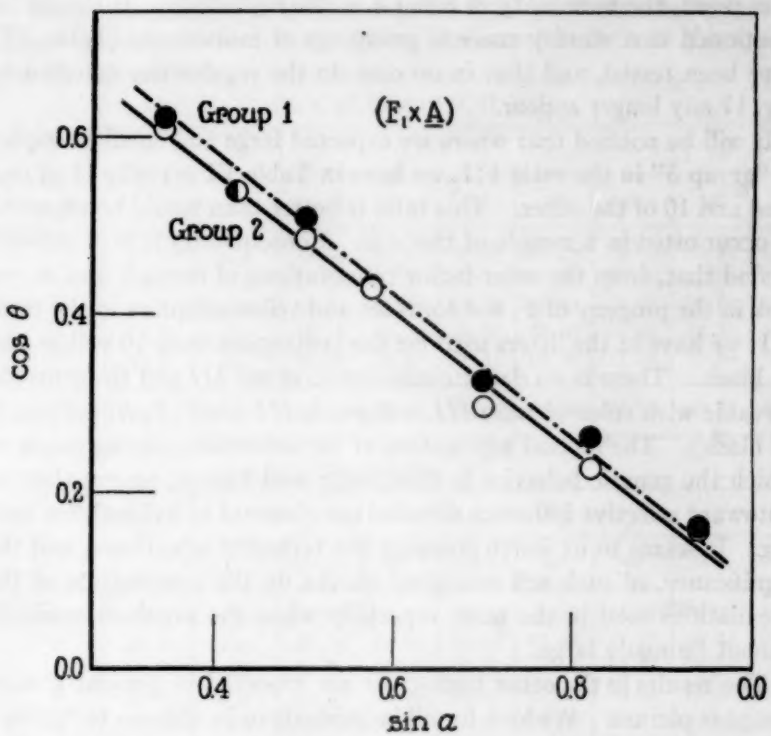

FIG. 18. *Cos* θ vs. *sin* α for the two groups of individuals in the families ($F_1 \times A$). For one of these, the line should be similar to that for F_1; for the other, it should be similar to that found for the *A* grandparents. The group of composition (i, ii, iii) give a slope $\Delta \cos \theta / \Delta \sin \alpha = 7.8$, with $\Delta \sin \alpha$ taken = 0.4, while for the *A* line the slope is 7.8 (*cf.* Fig. 1). [The fact that the position of the line is slightly lower on the *cos* θ axis is discussed subsequently in the text; this effect causes the θ – *log sin* α plot (Fig. 17) to be as it were rotated about a mid-point (by comparison with that for *A*), and thus to signify a broadening of the distribution curves for thresholds of our groups of sense organs.] The group of individuals presumptively (i, ii, III) in constitution should on the present coördinates show a break in the graph, with increased slope at the high α–end. The best fitting straight line should have a slope similar to that already gotten for the similarly constituted F_1 (Fig. 5); it is actually = 8.0 units, as compared with 8.4 units for F_1.

sin α for the two groups of individuals are given in Fig. 18. (At a later point, the variability of mean θ is also discussed). It should be mentioned that sundry random groupings of individuals (Table VI) have been tested, and that in no case do the regularities detected in Fig. 17 any longer appear.

It will be noticed that where we expected large and small examples of "group 3" in the ratio 1:1, we have in Table VI actually 11 of one kind and 10 of the other. This ratio is better than would be expected to occur often in a sample of this size. Consequently it is of interest to find that, from the color-factor constitutions of races *K* and *A*, we look in the progeny of F_1 x *A* for *black* and *yellow* offspring in the ratio 1:1; we have in the litters used for the geotropism tests 10 yellow and 11 black. There is no definite association of our *III* and *iii* forms observable with color (4 were *III*, yellow; 6, *III* black; 6, *iii* yellow; 5, *iii* black). The factual segregation of the color-determining genes, of which the genetic behavior is sufficiently well-known, proves that no untoward selective influence dictated our choice of individuals for testing. It seems to us worth stressing the technical advantage, and the significance, of such self contained checks on the composition of the populations used in the tests, especially when the numbers available cannot be made large.

The results in the other back-cross are expected to present a more complex picture. We look for all individuals to be alike as to "group" III; but, if free assortment of whatever it be that determines the I, i; II, ii association is to take place, we look for *four* groups of individuals phenotypically separable, showing all possible combinations of the "large" and "small" groups in positions 1 and 2. Since it is physically out of the question to manipulate *large* numbers of progeny and yet to keep all necessary conditions uniform, and since our primary aim was to test the nature of differences between our original formulae for the *K* and *A* lines, *any* evidence of segregation is to be looked upon as favorable to the inquiry.

From the indications given by the distributions of θ at 20° (Fig. 16) we have already seen reason to expect in (F_1 x *K*) *two* groups of individuals, separable at low values of α. More complete separation is obtainable in two other ways. With sufficiently reliable measurements we might analyze the θ-log sin α curve for each individual, grouping

TABLE VIII

Individuals produced in the backcross ($F_1 \times K$) are empirically separable, as regards $\Delta\theta/\Delta\alpha$, into four distinct groups, as discussed in the text; *bl.h.* = *black hooded*, *alb.* = *albino*.

($F_1 \times K$) Group 1 (I, II, III)

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
4	21.5	*bl.h.*	49.06	56.32	60.88	63.63	79.00	80.38	84 68
5	19.5	*bl.h.*	47.50	57.29	60.36	61.08	76.29	81.00	82.71
8	20.5	*alb.*	50.52	53.04	59.45	68.44	71.17	80.84	83.59
11	19.0	*alb.*	48.88	53.25	58.90	68.99	74.63	82.53	84.74
19	20.5	*alb.*	49.23	56.23	58.41	68.95	77.94	75.50	86.67
Mean =	20.2		49.23	54.63	59.48	66.94	74.98	80.51	84.53

Group 2 (i, ii, III)

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
1	22.0	*alb.*	56.13	61.15	63.17	64.33	74.07	74.83	81.55
9	20.5	*alb.*	52.75	53.04	59.45	68.44	71.17	80.84	83.59
10	19.5	*alb.*	53.76	56.48	60.41	67.30	72.54	76.32	77.05
12	20.5	*bl.h.*	53.18	59.58	63.08	67.41	70.48	77.76	83.57
13	19.3	*bl.h.*	51.79	57.83	61.59	65.36	70.21	76.11	82.29
21	17.5	*bl.h.*	56.52	58.93	62.47	66.50	76.80	77.44	81.90
22	20.4	*alb.*	54.63	57.43	61.06	67.38	74.61	81.05	81.57
Mean =	20.0		53.66	57.40	61.44	66.84	72.21	77.62	81.64

($F_1 \times K$) Group 3 (I, ii, III)

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
3	21.2	*bl.h.*	51.13	56.58	61.58	56.67	72.46	73.50	78.09
6	20.0	*alb.*	52.13	56.73	62.59	67.25	73.31	80.57	83.65
7	17.0	*alb.*	47.96	55.92	58.82	69.58	68.89	78.17	79.04
15	30.0	*alb.*	51.96	57.00	61.17	66.11	71.55	77.63	83.88
18	28.0	*alb.*	49.11	56.86	57.68	65.44	75.94	75.59	81.29
Mean =	23.2		50.34	56.53	60.60	65.98	72.05	77.74	81.23

TABLE VIII—*Concluded*

($F_1 \times K$) Group 4 (i, II, III)

No.	Wt., gms.	Color	θ						
			$\alpha = 20°$	25°	30°	35°	45°	55°	70°
2	22.8	*bl.h.*	53.46	60.36	61.58	62.88	75.50	79.91	82.00
14	26.0	*alb.*	53.93	55.82	61.94	65.95	75.06	76.00	79.44
16	28.5	*bl.h.*	54.12	57.75	—	64.93	69.84	77.39	78.33
17	26.5	*alb.*	52.87	55.03	59.38	63.82	73.76	78.94	79.10
20	19.5	*bl.h.*	53.28	59.53	61.21	64.68	76.05	—	82.25
Mean =	24.8		53.53	57.69	61.03	64.65	74.04	78.06	80.22

these in accordance with the indications of the presence of I, i; II, ii, or the other combinations. This has in fact been done. The alternative method is to consider the *cos* θ − *sin* α plots. The results of these two methods are completely concordant. Limitations of space lead us to present only the result of the latter proceedure in detail,but the material for the former treatment is given in Table VIII.

By very fortunate chance the races initially chosen for these experiments, *A* and *K*, turn out to be rather "simple", in the sense that our three "groups of sense organs" are each large, in *K*, small in *A*. Were it not for this fact the analysis would be much more difficult. As one result of the actual condition, the cos θ — sin α plots for these races (Fig. 1) are straight. But in a combination corresponding to I, ii, or to i, II, the graph with these coördinates should show a sharp break. With I, ii, the low - α end should be steep, as in the *K* race, the mid region less steep, as in *A* (Fig. 1). None-the-less, recognizing possibilities of errors in the determinations of θ with any one individual taken singly, it is possible to draw a fairly satisfactory line through the points for each one, plotted as cos θ *vs.* sin α. In the case of the *K* race, the slope of this line, in arbitrary units [4.0 Δ cos θ/(Δ sin α = 0.4)] is 12.6 units; for the *A* race, 7.8 units; for F_1 (*K* x *A*) it is 8.4 units. We expect in (F_1 x *K*) a group of individuals showing a cos θ — sin α slope near to 12 units, another with slope near 7.8; and, if the previous assumptions are just, two other groups with slopes in between these values. The relatively greater uncertainty (experimentally, not statistically,) of θ at $\alpha = 70°$ makes the finer separation

doubtful, since this θ is likely to be low. At least, we can say that one group should have the slope $\Delta \cos \theta/\Delta$ in α (chiefly weighed by the θ's at $\alpha < 70°$) near to that for K; another, more numerous, near to that for A; another, about as numerous as the first, with the slope intermediate. In fact the grouping by this method is rather rough,

TABLE IX

$(F_1 \times K)$

Association of individuals in $(F_1 \times K)$ as determined chiefly by the slope $\Delta \cos \theta/\Delta \sin \alpha$; as explained in the text, this is a rough measure only, and has to be corrected according to other criteria.

No.	$\frac{4.0\ \Delta \cos \theta}{\Delta \sin \alpha = 0.4}$	No.	$\frac{4.0\ \Delta \cos \theta}{\Delta \sin \alpha = 0.4}$
Group 1 (I, II, III)		Group 2 (i, ii, III)	
4	9.5	1	6.8
5	10.1	9	7.7
8	9.8	12	7.8
11	11.1	13	7.6
19	9.9	22	8.2
		21	7.1
Mean =	10.1	10	7.6
		Mean =	7.6
Group 3 (I, ii, III)		Group 4 (i, II, III)	
3	8.8	2	8.1
6	9.2	17	8.1
7	9.0	20	8.0
15	8.1	14	8.2
18	9.0	16	6.7
Mean =	8.8	Mean =	7.8

although quite clear, as Table IX shows; it has been checked by the more detailed consideration of the $\cos \theta - \sin \alpha$ plots for each individual, where it is clear that the slopes for the parts of the graphs compel the recognition of independent assortment of I, i and II, ii "groups of sense organs." This mode of associating the individuals is exhibited in Table VIII. The mean θ's for each group are plotted in

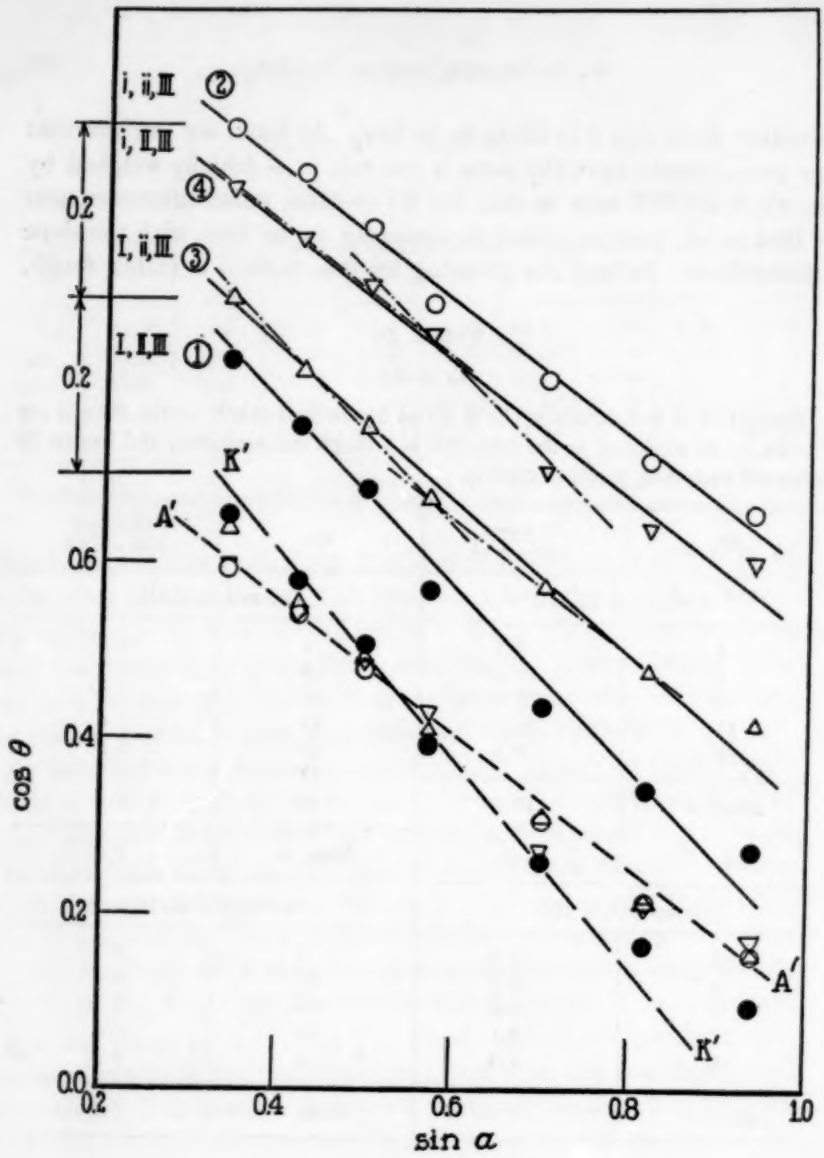

FIG. 19. Cos θ *vs.* sin α for four groups of individuals separated in the progeny obtained from ($F_1 \times K$). The slopes of the fitted lines are those given in Table IX (see text). In the lower part of the Figure the points are plotted *en masse*, and (with the shifts, A', K', discussed in the text) the lines for the grandparental families A and K are seen to include between them the scatter of all the data from the back-cross segregates. In the upper portion of the Figure the four groups of back-cross individuals are treated separately. The probable errors are less than the diameters of the symbols. In fitting the unbroken lines, relatively less weight has been given θ at $\alpha = 70°$; this point is the most likely to be untrustworthy.

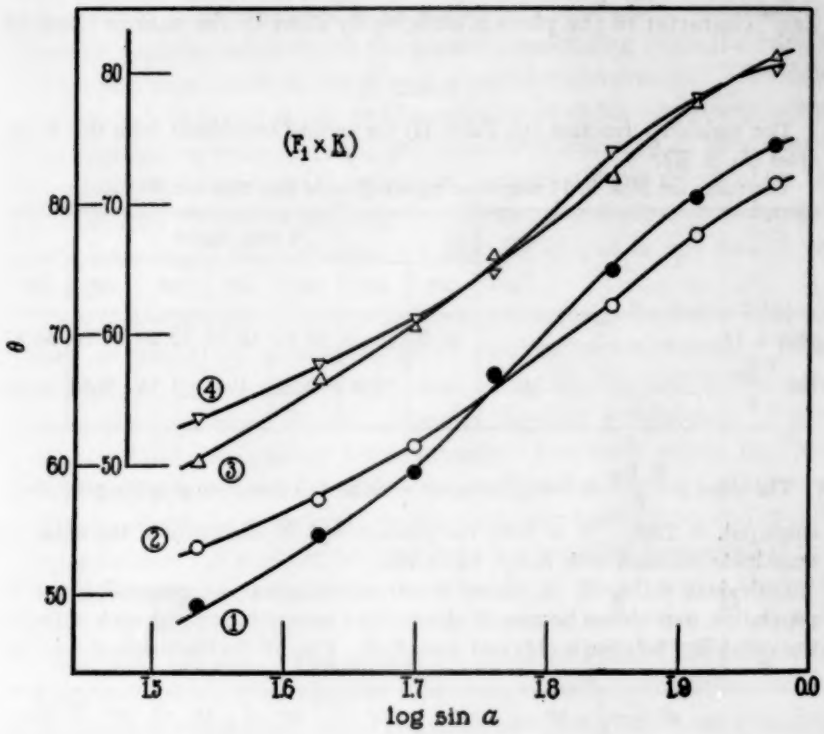

FIG. 20. θ vs. log sin α for the four groups of individuals segregated in the back-cross progeny ($F_1 \times K$). Group 1 should be like the grand-parental *K;* Group 3 should have its low-α end like that for 1, its mid-portion like that of Group 2. Group 2 should resemble closely, except at $\alpha = 55° - 70°$, the grand-parental *A*, or more closely the Group (i, ii, III) of segregates in the back-cross ($F_1 \times A$), with which its curve is indeed identical. Group 4 should show a lower portion of its curve like that in the curve for Group 2, its later portion like that in the curve for Group 1. In other words, as regards the slopes of these curves, considering each curve divided into two parts on either side of $\alpha = 35°$, we should find all possible combinations of low and high slopes—high slope below 35° combined with either high or low slope above 35° (up to $\alpha = 55°$), and reciprocally. It is clear that these combinations are indeed found.

Fig. 19, where the slopes given to the lines are those obtained in Table IX. When the separate series are studied it is evident that the "broken" character of the plots is sufficiently clear in the case of three of

TABLE X

The variability function (*cf.* Table II) for certain individuals from the backcross ($F_1 \times K$).

The value for Nos. 8, 11 might be expected to be like that for *K:*

	θ, mean, degrees						
α =	20°	25°	30°	35°	45°	55°	70°
Nos. 8, 11	49.70	53.15	59.18	69.72	72.90	81.69	84.17
$100 \frac{\text{P.E.}_\theta}{\theta}$	2.7	2.0	2.21	1.42	1.14	0.80	0.63

The slope $\Delta \frac{\text{P. E.}_\theta}{\theta} \times 100 / (\Delta \log \sin \alpha)$ is in this case, using units previously employed, = 2.00; N = 3.88; the product = 7.76, obviously of the order of magnitude obtained with *K* (*cf.* Table II).

Individuals 9, 10, 12, 13, placed in our second group, *i.e.* resembling the F_1 population, were chosen because 20 observations were obtained with each of these; the variability function is obtained from P. E. $/\theta \times 100$ for the summed records:

α =	20°	25°	30°	35°	45°	55°	70°
100 P.E.$_\theta/\theta$ =	1.67	1.46	1.56	0.60	0.72	1.08	0.75

is $0.9 \times 0.71 = 0.64$

This compares sufficiently well with the value 1.92 (Table II) gotten with F_1, as to order of magnitude.

The cogency of this reasoning is further supported by analysis of the variability of the group of ($F_1 \times A$) individuals (Table II), determined to be like race *A*. For these, the value obtained is 2.5, which as to order of size agrees well with the value for *A* as gotten in Table II.

the four sets. The remaining one is like the original *K* line, save that the θ's are uniformly higher (cos θ's less). This corresponds to a uniform lowering of the *K* graph, with only very slight change of slope. The same shift is seen in the graph for the *A* line which "ought" to

describe the course of the observations with the second set (i, ii)below $\alpha = 50°$. It will be noticed that the graphs for the four sets cross at $\alpha = 36°\pm$, as predicted in an earlier Section (p. 78), and that the mean θ's are included within the lines corresponding (with the parallel shifts just mentioned) to the *K* and *A* grandparental races. The shifts correspond, it seems to us, to the entrance of other (genetic?) factors not segregating with I, i, etc., but capable of affecting θ; they cannot be associated simply with weight of individual; the effect upon the distribution curves for $\Delta\theta/\Delta \log \sin \alpha$ is to cause them to broaden out, with lowering of the ordinates (*cf.* Fig. 20, 21), as in the case of our *B* race.

An important check upon the associations given in Table VIII has been obtained by various trials at random and obviously "false" groupings. It was pointed out much earlier (p. 78) that in the total (F_1 x *K*) population P. E. $_\theta/\theta$ passes through a minimum at $\alpha = 36°\pm$, plainly indicating heterogeneity. For each group in Table VIII the variability of θ declines as a straight-line function of log sin α. With sundry false groupings this is at best only feebly true, and usually quite untrue. A striking point about the groupings here given is that for the segregates expected to be like the *K* grandparents the variability is so likewise; while for those expected to be like F_1 (*K* x *A*) this is also the fact. The *average* θ's given in Table VIII include for some individuals $20\pm$ observations each at each α, for others 40 $\pm$; the latter were obtained in the course of tests designed to see if facilitation ("learning") influenced the measurements of θ — as we have already discussed (p. 60). To simplify the presentation we choose two individuals of the group I, II. III and four from i, ii, III, and in Table X give the variability function for the two lots. If the *variability* is a function of specific sense organ groups, the "variability number" for the first lot should be like that for *K*; of the second lot, like that for F_1 (*K* x *A*). Table X shows that to a remarkable degree this is indeed the fact, although we do not seek to stress the conclusion unduly until further data are available.

If there is free association of I, i, II, ii, and of the color-factors involved in the cross, we expect equal numbers of I and of i, of II and of ii, and of *black hooded* and of *albino* individuals, with no indication of connection between color and geotropic performance. Of I and i

there are in Table VIII respectively ten and twelve cases; of II, ii, respectively ten and twelve; of the former (*cf.* Table VIII) nine are *bl.h.*, thirteen *alb.;* of the latter, the ratio is the same. Table XI shows no connection between *color* and geotropic response. As stated in discussing F_1 (A x K), this fact we regard as direct evidence that no untoward influence dictated our selection of litters for examination, since the inheritance of the color genes is well known, and the appearance of the patterns in our individuals shows chance arrangement.

When θ is plotted against log sin α, we expect for these groups of individuals curves of which the differentials should show, when plotted

TABLE XI

Distribution of the "sense organ groups" I, i; II, ii in relation to color factors, in the progeny ($F_1 \times K$).

	bl.h.	*alb.*	
I	3	7	*10*
i	6	6	*12*
	9	*13*	
	bl.h.	*alb.*	
II	5	5	*10*
ii	4	8	*12*
	9	*13*	

against sin α, the 3 "groups of sense organs" previously recognized. Of the θ-curves we expect one to be like that for F_1, one to be like that for K, the other 2 different in certain predictable ways. At the same time, the shift of the cos θ graphs we have earlier remarked as "flattening" the differential curves. This corresponds to a curious "rotation" of the θ — log sin α curve, about a mid-point, as seen also in the comparison of lines A and B, and may be related to the participation of other ("genetic"?) influences, but we are in no position to discuss these concretely. However, there is no difficulty in recognizing the four kinds of individuals we in this case seek. It is instructive to notice

that several of these sorts of individuals give θ — log sin α graphs much more nearly straight, as a first approximation, than those originally gotten for races *K*, *A*, *B*, etc. This again reminds one of the dangers of "Weber's Law," and leads us to remark that this particular straight-line is not quite the same thing as that usually appealed to in such relations. In the present cases the approximate rectilinearity holds over the whole workable range of α, whereas it is well known that the relation usually seen between response and log stimulus, and for photic excitation clearly analyzed by Hecht (1924, *b*), holds only over the midrange.

Curves of θ *vs.* log sin α for the four sorts of individuals recognized in (F_1 x *K*) are plotted in Fig. 20. The differences among these have already been briefly characterized. It may be noted that the probable errors of the mean θ's are less than the diameters of respective symbols, but are a little larger than might be expected on the basis of the numbers of readings, owing to the fact that the θ curves for two individuals may be parallel, but $\Delta\theta/\Delta\alpha$ the same; the use of litter-mates in the *K* and *A* series apparently avoided this effort.

A general scheme for the inheritance of these phenomena can accordingly be written:

Receptor groups	1	2	3
Race A	i	ii	iii
Race K	I	II	III
F_1, A × K	i(I)	ii(II)	III(iii),

with the realization that in backcrosses with *A* and with *K* the expected phenotypic classes, recognizable by several different tests, in each case appear to be found, and in proportions of individuals which clearly indicate the possibility of independent alternative inheritance of the factors recognizably concerned.

Genetically, the amount of geotropic orientation in these animals must be regarded as a "multiple factor" effect. It is possible to foresee that in certain other instances it may be feasible to disentangle the relations of several cooperating genes by the similar application of methods which seek to modify quantitatively the expression of composite resultants according to the influence of some controlling condition of the respective contributing factors.

VI

With regard to the possible significance of these differences, interpretation must proceed slowly. They cannot be accounted for by mere differences among the weights of the several individuals. Since, as we have seen, the values of θ at threshold α are apparently distributed in a way corresponding, at least roughly, to the finer differences brought out, it might be conceived that *re-distributions* of the weight, in terms of differential growth rates of the regions of the body, might influence in a differential way the excitation of a fundamentally uniform series of receptors,—essentially the same, that is, in all the cross-bred individuals. But a little reflection makes it appear that this assumption would require rather elaborate hypotheses if it should attempt to explain in detail the effects as found, and it could not interfere with the conclusion we are impelled to draw.

If we accept the initial assumptions of these experiments, chiefly that according to which θ is determined by the achievement of essentially equivalent excitation of tension receptors on the two sides of the body, θ in degrees being therefore a proportional statement of the total amount of excitation, we must recognize at least the following elements in the case: (1) the magnitudes of the exciting pull ($k \sin \alpha$); (2) the variation of excitation thresholds among the population of receptors; (3) the frequency with which their excitation occurs;and, presumably, (4) central nervous thresholds. The relative simplicity of the results obtained in endeavoring to analyze the observations encourages us to believe that the method of treatment deals successfully with (1) and (2–3). It is implicit in the treatment that by a "receptor" effect we include the whole chain of events from sense organ through to central organ and beyond, and include also the frequency of excitation,—because it is in these terms that our "groups of receptors" are recognized. But it should be noted that the speed with the A line is, at given α, a little *less* than with K; qualitatively it increases in the same way. We have ignored possible differences in speeds of creeping, because no great absolute differences were perceptible between the several lines used; and there is at present no means of dealing with the significance of the speed as related to length of limbs and the like. The matter of "central thresholds" ((4) above,) we cannot deal with directly. In view of the correlation between

threshold θ's and the slopes of the θ — log sin α lines, it might be supposed that the essential genetic differences between lines, *K*, *A*, *B*, and the *K* x *A* hybrids relate merely to the ease with which particular assemblages of tension-receptors become effective as loci of stimulation, whether through differences in central nervous conditions, or through differences in the relative excitabilities arising through diverse arrangements of the body mass relative to the dimensions or attitudes of the appendages during creeping, or to the frequencies of stepping. Such differences might well be responsible for the fact that at the slope where θ begins to be exhibited as a definite function of the slope, different "numbers of receptors" are already involved in the several cases. The question remains, whether these additional receptors are drawn from the whole available array, or selectively from one or more groups of them. But in neither case would the effect in any way minimize the significance of the recognition in the present cases of *three* such groups. As is shown in the final section of this paper, a selective effect on *one* of these three groups can be demonstrated when an additional mass is appropriately carried by the creeping rats. A choice among the several alternatives suggested (and others are possible) would affect merely the *interpretation* of the genetic results. In other words, we cannot say that the genetic differences utilized and revealed in the present experiments definitely depend upon the occurrence of three distinct groups of sense organs, the number of organs in each group differing in the several pure lines used and being subject to the rules of simple alternative inheritance, *because the conditions of their effective excitation may be the things subject to inheritance.* Whether these conditions are of a central nervous sort, or depend merely upon the relative rates of development of the numbers of tension receptors in our three groups, in the several races used, cannot be stated. The contrast between lines *B* and *A* might be understood in either way. These considerations do not affect the fact (1) that the quantitative differences found between lines *A* and *K* are shown by their genetic analysis to be *real* differences, and (2) that genetic differences of the sort we have used can be recognized and characterized only by quantitative procedures which seek to define the genetic phenomenon as a function of an experimentally controllable variable.

VII

It has been clear to us that the existence of the three "groups of sense organs" apparently concerned in the adjustment of the geotropic orientation of our young rats might be made more certain if demonstrable by some independent test. At the same time, it might be possible to predict upon this basis, at least in a *general* way, the outcome to be expected as result of certain modes of experimental manipulation. If, for example, our three categories of sense organs inhabit diverse organic loci,—muscles, tendons, skin,—it might be possible by increasing the loads to be carried by the young rats to modify in a

FIG. 21. Outline of a rat creeping on an inclined surface, with flat brass weight attached to the skin of the back. Outline from motion-picture record.

predictable way the curve connecting θ with α. We have earlier demonstrated (Crozier and Pincus, 1926–27,*a*; Pincus, 1926–27) that when small masses are attached to the base of the tail of these animals, at constant α there is evident an increase of θ about proportional to log m, where m is the added mass, and that the *speed* of linear progression is similarly influenced. The information now required, however, depends upon measurements of θ as a function of α, with m constant. If the added weight is so located as to influence primarily *one* of our "three groups of sense organs," bringing it into play at lower values of α than in the absence of the weight, then we expect the curve connecting θ with log sin α to be distorted at one end or the other. In that event, curves of the type of those already

shown in Figs. 10, 11, should undergo a change signifying a shift of *apparent* thresholds of the sense organs of one or more groups.

An experiment of this sort may be described here. It was made with individuals of race *K*, under standard conditions, the individuals first being tested for orientation at several values of α and then employed after a flat brass weight (2 gms.) had been attached to the center of the back with a thin layer of chicle. The total attached mass was

TABLE XII

Angles of upward orientation (θ) of young rats of line *K*, carrying a mass of 2.15 gm. as shown in Fig. 21, at various inclinations of the surface (α). Two series of tests (i, ii), with four individuals ($n = 40$) in each. The way in which Θ is increased, at lower values of α, is made clear by Fig. 22. The manner in which the $\Delta \cos \theta / \Delta \sin \alpha$ relationship is disturbed is shown by Fig. 24.

α degrees	θ degrees	
	(i)	(ii)
15	51.56 ±1.38	49.80 ±1.20
20	59.65 ±1.32	57.76 ±1.09
25	66.80 ±1.07	63.71 ±0.92
30	70.10 ±0.94	
35		69.20 ±0.87
40	70.83 ±1.01	
50	78.41 ±0.80	
55		78.91 ±0.71
65	85.15 ±0.55	
70		86.12 ±0.43

2.15 gm. It is not particularly easy to attach weights to these animals, on account of the looseness of the skin, and the method now used was adopted only after trial of a number of others involving the use of tiny harness straps (of rubber dam), clamps, and the like. The diagram in Fig. 21 illustrates the relation of the weight to the animal's axis.

The experiment comprised two parts. In the first, measurements of orientation were obtained at various inclinations in about 10 runs with each of four individuals. Care was taken to exclude fatigue

effects. The second part of the test was a repetition of the foregoing with a new litter of rats, some days later.

The results from these two experiments are collected in Table XII. The plot in Fig. 22 makes it evident at once that at low values of α, θ is increased, in a regular manner, by the presence of the added mass.

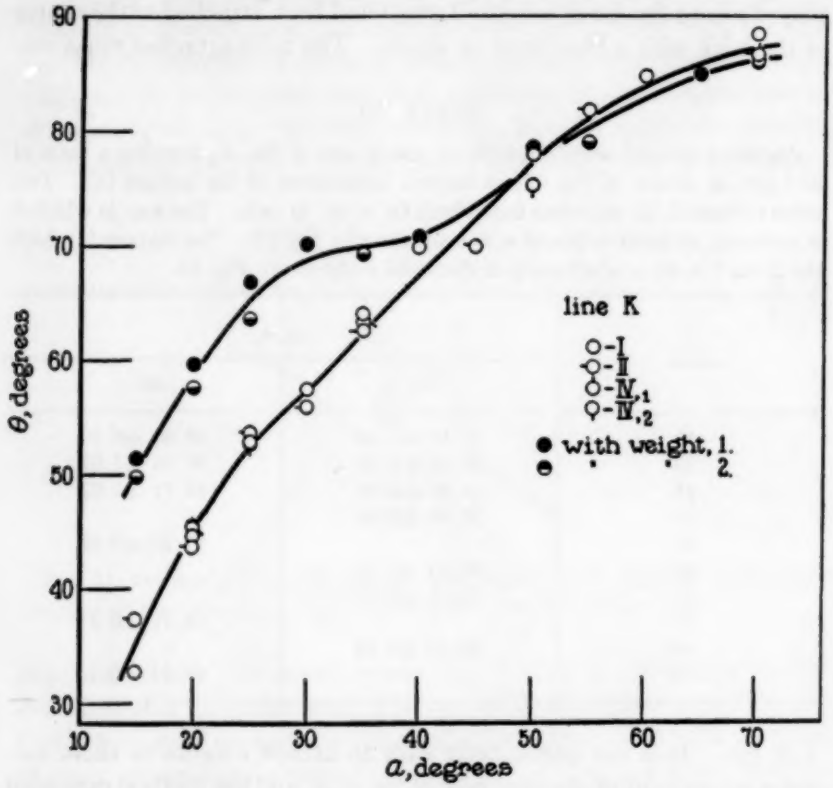

FIG. 22. Orientation-angles (θ), at different slopes of surface (α), for individuals of line *K* with added load (2.15 gms.); lower curve, without weights.

Moreover, the flatness of the curve at intermediate magnitudes of log sin α clearly suggests the kind of unequal distortion of the θ curve which we have seen reason to expect. The differential curves corresponding to the θ-graphs in Fig. 23, obtained as previously described in connection with Figs. 10, 11, are given in Fig. 25. If the amount and the adjustment of the weight in these tests has been such as to

influence *chiefly* the sense organs of our group II, and to a lesser extent those of group I, bringing the affected ones into play at low angles of inclination, below or as low as at the working threshold inclination, then the area under the differential curve *B* in Fig. 25 should be less than, and proportionately related to, the area under curve *A* in Fig. 25. It is evident that this is in fact the case. The mean θ, without

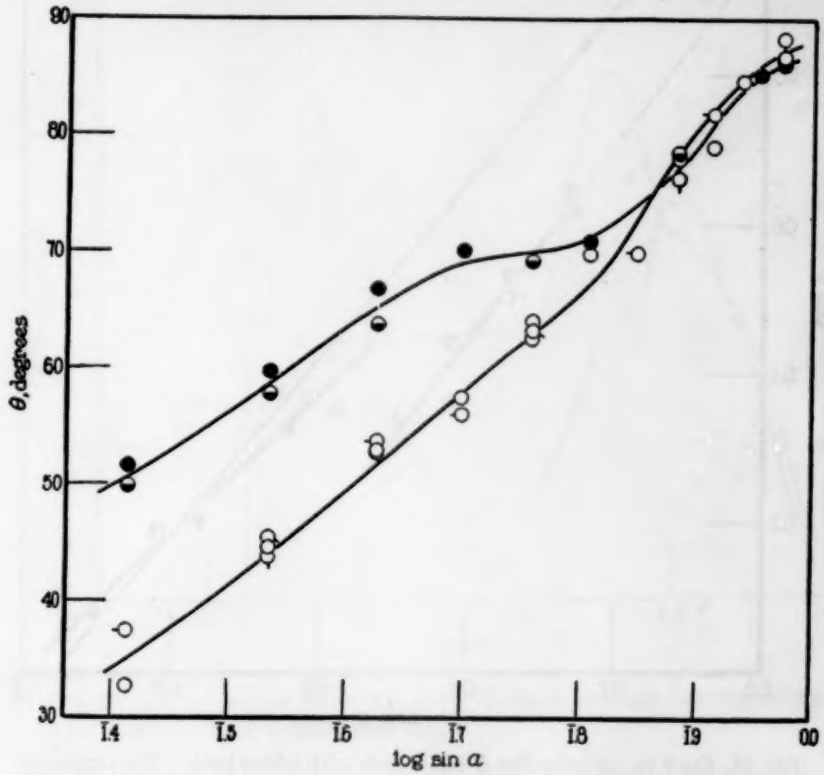

FIG. 23. θ vs. *log sin* α, with and without added load (2.15 gms.); race *K*.

weight added, at 15°, is (from the curve) 34°; with a load of 2.15 gms., $\theta = 50°$. Continuing the assumption that $\theta \equiv$ the total number of sense organs capable of controlling the geotropic orientation, and assuming that *all* these are activated when $\theta = 90°$, then the number remaining to be activated by increasing α above 15° is, without the weight, proportional to 90−34, or 56; with the load, to 90−50, or

40; in the latter case the number of "available sense organs" is then reduced in the proportion of 40/56, or of 2.0/2.8. Actually, the areas under the two curves in Fig. 25 are (in arbitrary planimeter

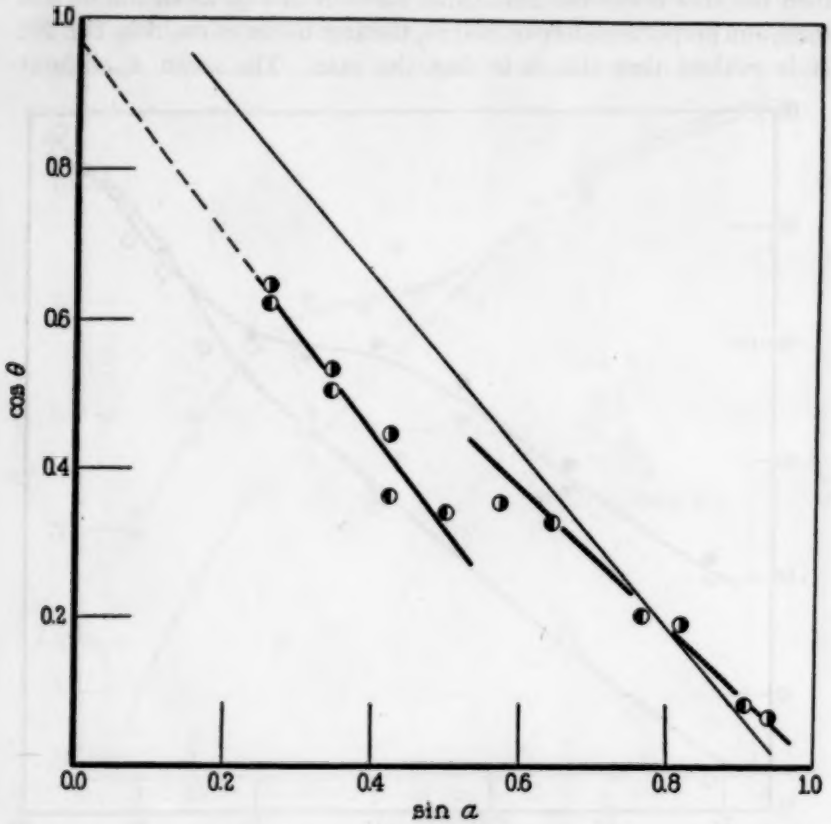

FIG. 24. *Cos* θ vs. *sin* α for line *K* individuals with added load. The original *K* graph is added as a thin line. Note that the projection of the initial segment of the broken graph goes practically through the origin ($\alpha = 0, \cos\theta = 1$); the downward displacement is a function of the added mass.

units) as 24.90 to 16.77, or in the ratio of 2.0/2.98. If we assume that all the activation effectively possible is obtained at $\theta = 83°$, then the ratio (83 − 34) / (83 − 50) is as 2.0/2.97. This *is* effectively the maximum obtainable θ. The agreement is so close as to preclude accident,

we believe; if this be correct, there results a striking justification of the conception that the orientation angle is determined in such a way as to reflect, directly, the total number of receptors activated per unit of time during oriented progression. It will be noted that on the *cos* θ plot (Fig. 24) the extension of the line appropriate to the observa-

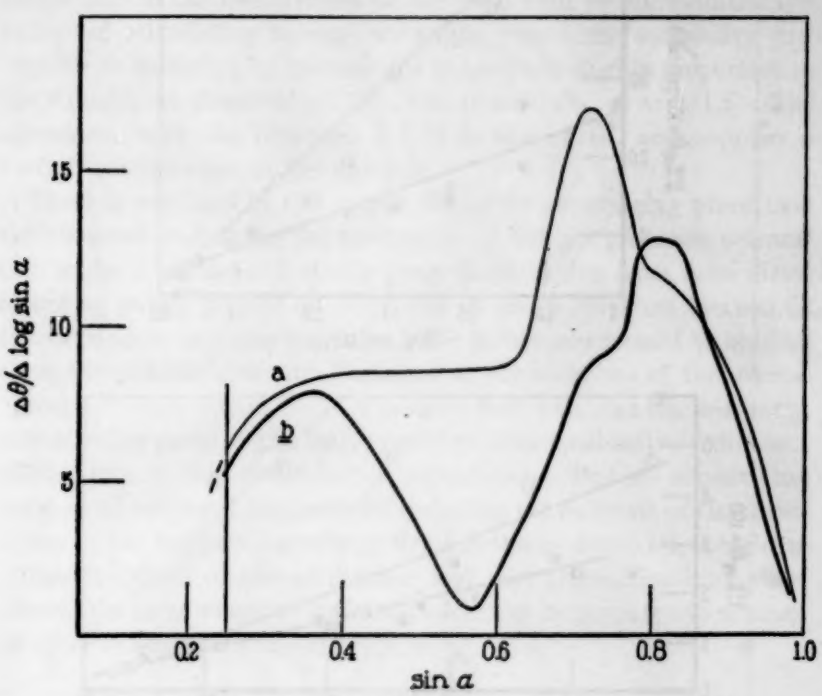

FIG. 25. $\Delta\ \theta\ /\ \Delta$ log sin α, vs. sin α, for *K*-line individuals carrying weights (*a*); for *K*-rats without weights, thin line.

tions at lower values of θ passes practically through the $\theta = 0$ origin (*cos* $\theta = 1$). This would be interpreted to mean that the presence of 2.15 gms. added mass stimulates none of the receptors in question while the animal is moving in a horizontal plane. The modifications of the curves which might be expected with larger masses, or by employing rats of other races, cannot be entirely foreseen.

The variability of θ, as P.E. $_\theta/\theta \times 100$, is much reduced, propor-

tionately, at equivalent α, for individuals carrying an added load (2.15 gms.), as shown in Fig. 26 A. By comparison with a series (open cir-

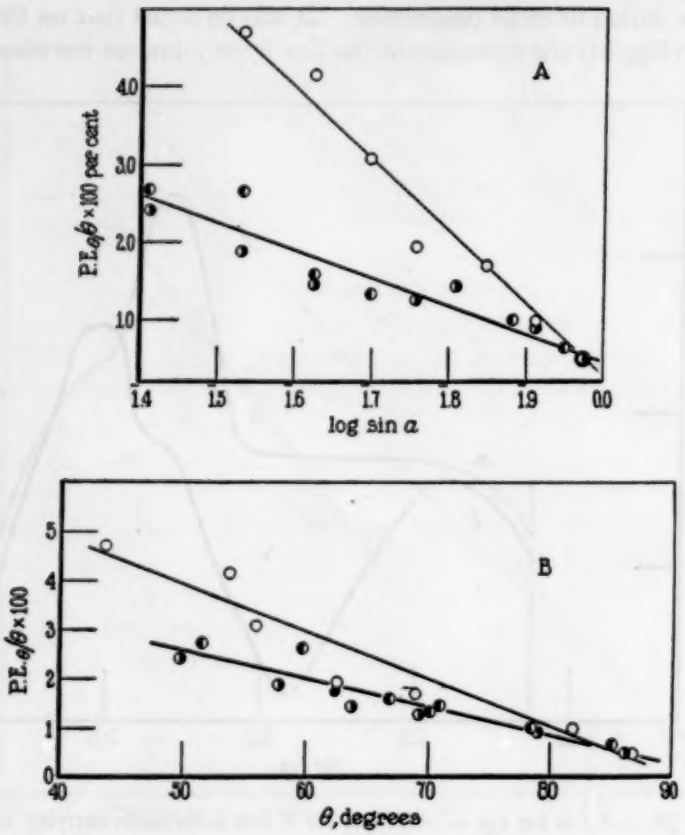

FIG. 26. *A*. The percentage variability of θ, as P. Γ $/\theta \times 100$, is much reduced, proportionately, at equivalent α, for individuals carrying an additional load (2.15 gms.), by comparison with series *K* II (open circlets) where the same numbers of individuals and of observations are concerned.

B. The variability of θ plotted against θ, for the two sets of observations. See text.

clets) in which the same number of individuals (4) was concerned, with the same number of readings on each, the slope Δ (P.E./θ)/Δ

log sin α is in the two cases in the ratio of about 1:2.57. If the probable errors, as percentages, are however plotted against θ for each series, as in Fig. 26 B, the relationship is again sufficiently rectilinear, and the comparison is fairer. The slopes of the fitted lines are as 1:1.66. If we assume the variability, with the load, to be reduced in proportion to the effectiveness of the total load as a gravitationally activated stimulating agency, we might expect the variability susceptible to reduction by increasing α to be diminished in proportion to the θ angles at threshold α (15°); this is as 51:34, or as 1:1.5. The agreement with the observed 1:1.66 is suggestive, and supplies a further confirmation of the analysis.

There is obtained in this way a distinctly encouraging proof that the elements underlying the assumption of definite geotropic orientation under conditions of steady progression, which have been dissociated by purely genetic methods, can by an independent method be shown to occur as distinct entities. We do not now hazard an opinion as to the possible structure character or the locations of the diverse "groups of sense organs." It is possible that weights attached to the rats at other parts of the body would produce qualitatively different effects; indeed, this would be our expectation. But we submit that the general results of this analysis, including the outcome of the application of the independent tests which have been described, constitute a reasonably self-consistent picture; and that alternative interpretations of the facts which we have adduced must be prepared to account for these relationships in a definite way.

VIII

SUMMARY

1. Equations describing the geotropic orientation of young rats as a function of the inclination of the surface on which creeping take place, under standardized conditions, are found to be of similar form but with different values of the contained constants, when several different, genetically stabilized lines or races are compared. The values of these constants are characteristic for the several races.

2. The biological "reality" of the differences between young rats of two races, as given mathematical form in terms of these parameters

and coefficients, can be submitted to radical test by investigating their behavior in inheritance. A simple result favorable to the inquiry would be decisive; a complex, non-clear result would not however be definitely unfavorable to the view that "real" differences in behavior are in question. The actual result is of a kind demonstrating (a) the efficiency of the original formulations, and (b), at the same time, the definite inheritance of certain quantitative aspects of geotropic behavior.

3. On the assumption that orientation on a sloping surface is achieved when, within a threshold difference, the tension-excitations on the two sides of the body (legs) are the same, the angle of oriented progression (θ) can be taken as a direct measure of the total excitation. This is consistent with the equation, accurately obeyed by our initial races, $\Delta \cos \theta / \Delta \sin \alpha = -const.$, where α is the slope of the surface.

4. The total excitation of tension-receptors must be regarded as involving, over a gross interval of time, (1) the total array of receptors with thresholds below a certain value, a function of the stretching force, and (2) the frequency of change of tension. The latter, largely determined (it is assumed) by the frequency of stepping, should be proportional to the speed of progression. This speed is directly proportional to $\log \sin \alpha$. Hence $\Delta\theta/\Delta \log \sin \alpha$. plotted against $\sin \alpha$, should give a picture of the distribution of effective thresholds among the available tension-receptors in terms of the exciting component of gravity. For the races investigated this distribution can be resolved in each case into three groups.

5. A "variability number" is employed which permits the demonstration that the variability of θ as measured is (1) definitely controlled by α, and is (2) a characteristic number for each of the pure races used.

6. By attaching a weight to rats of one race it is found that $\Delta\theta/\Delta\alpha$ is modified in a manner concordant with the assumption that the three "groups of sense organs" are in fact discrete.

7. In race *K* these three groups (I, II, III) are large, in race *A*, small (i, ii, iii). F_1 rats of the cross between these two races show i, ii, III.

8. F_1 individuals back-crossed to *A* give in the progeny two sorts of individuals, in equal numbers: i, ii, III and i, ii, iii.

9. F_1 individuals back-crossed to *K* are expected to give in the progeny four types of individuals, I, II; i, II; I, ii; i, ii. In the numbers available these classes are reasonably clear, and occur with equal frequency.

10. It is pointed out that these considerations imply a mode of definition of a gene somewhat different from that commonly employed by tacit assumption; namely, a definition of the effect in inheritance as a function of some controlling, independent variable.

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PROTEIN COAGULATION AND ITS REVERSAL

The Preparation of Completely Coagulated Hemoglobin

By M. L. ANSON and A. E. MIRSKY

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(Accepted for publication, June 27, 1929)

Irreversibility has always been considered one of the most striking characteristics of the coagulation of proteins. Four years ago, our experiments with hemoglobin led us to suppose that coagulation is reversible (2).† More complete evidence that the coagulation of hemoglobin can actually be reversed is provided by the present experiments. This first paper describes the preparation of completely coagulated hemoglobin and shows that the denaturation and coagulation of hemoglobin are the same as the denaturation and coagulation of other proteins. The second paper of this series describes the preparation from completely coagulated hemoglobin of soluble crystalline hemoglobin which by all the tests tried has been indistinguishable from the original native hemoglobin. Other papers are concerned with (1) the properties of proteins coagulated at interfaces, (2) the preparation of so-called native globin and the reversibility of the coagulation of globin, and (3) some properties of the tissue proteins and their significance in such phenomena as muscular contraction.

Description of Denaturation. As has been shown by Hardy (7),

* Many of the experiments described in this and the following paper were done in the Biophysics Laboratory of The Cancer Commission of Harvard University. A brief account of these experiments was presented to the Society of Biological Chemists in 1927 (3). Our first preparation of crystalline hemoglobin from coagulated hemoglobin was described in our doctoral dissertations, University of Cambridge, 1926.

† Wu and Lin (15) have confirmed these early experiments but they disagree with our interpretations of them. Spiegel-Adolf (13) has recently presented evidence that the denaturation of serum albumin is reversible.

Chick and Martin (4, 5), and Sørensen (12), coagulation proceeds in two distinct steps. The first step, known as denaturation, is a change in the native protein brought about by heat, acid, alcohol and other agents,* which makes the previously soluble protein insoluble around its isoelectric point. The second step in coagulation is the precipitation of the insoluble denatured protein. Denatured protein although insoluble around its isoelectric point is soluble in acid or alkali. If, therefore, a protein is denatured in a nearly isoelectric solution, a visible precipitate results. But if a protein is denatured in acid or alkaline solution, no visible change results, until the solution is made isoelectric—when the protein is precipitated. The second step in coagulation, the flocculation of the insoluble protein, is, as has long been known, reversible, since the flocculated protein may readily be redissolved. The first step in coagulation, denaturation, has hitherto not been reversed. When a solution of the coagulum in acid or alkali is brought to the isoelectric point of the protein, the protein is again precipitated; it is still denatured. Denaturation, therefore, is the important process in the investigation of the reversibility of coagulation.

Denaturation is not a general disintegration of the native protein. Did denaturation involve general disintegration reversibility would be impossible. The theory that denatured protein is an indefinite, early breakdown product of native protein is made plausible by the fact that heat, acids, alkalies and some of the other agents that bring about denaturation also cause a general disintegration. Heat, for instance, splits off nitrogen. Sørensen (12), however, has decisively shown that this splitting off of nitrogen is incidental rather than essential to denaturation. Similarly, we have found that alkali denatures a protein thereby rendering it insoluble in water, but that this same alkali gradually alters the denatured protein so that it becomes more soluble in water. It will be shown in a future paper that a protein solution made more viscous by alkali denaturation is (after denaturation is completed) gradually made less viscous by the alkali. These secondary effects of the denaturing agents should be clearly distinguished from the denaturation itself.

* It has not yet been proven that the various forms of denaturation result in identical changes in the protein.

I. The Preparation of Completely Coagulated Hemoglobin

It must be shown that the denatured hemoglobin used in our experiments on the reversibility of coagulation is completely denatured, that it contains no significant impurity of unmodified native protein. The ultimate test for complete denaturation is by definition complete insolubility at the isoelectric point. The possibility must be considered, however, that completely precipitated protein may be only apparently completely insoluble, for it is conceivable that denatured protein when precipitated carries down soluble native protein. Experimentally, however, at least under such conditions as render it possible to have a known mixture of native and denatured proteins, the precipitation of the denatured protein does not result in the removal from solution of any native protein. Furthermore, in the case of heat denaturation the kinetics of the reaction make it improbable that soluble protein is being carried down by the coagulated protein. In addition to solubility observations, there are two independent tests for complete denaturation. First, the same yield of soluble apparently native hemoglobin is obtained from hemoglobin denatured by heat at two different temperatures. This could hardly be true did the soluble hemoglobin come from any hypothetical residue of undenatured protein. Secondly, the denaturation of hemoglobin in urea solutions is accompanied by viscosity changes. When the viscosity ceases to change, the denaturation is probably complete.

In the following preparations of denatured hemoglobin the protein has been denatured in four different ways, by heat, acid, heat together with acid, and urea. In all four cases the completeness of denaturation has been tested by solubility observations. Wherever possible, the independent tests have also been used.

It is important to note that 30 per cent of the coagulated hemoglobin is finally obtained in the form of soluble, crystalline, apparently native hemoglobin. The existence of a trace of native hemoglobin in our preparations of coagulated protein would be insufficient to account for such a result.

(*1*) *Acid.* The first experiment describes the test for complete insolubility. The evidence is then given that native protein is not carried down.

1.3 cc. of a 10.85 per cent solution of horse hemoglobin are warmed to 40°C. To it are added 2 cc. $\frac{N}{5}$ HCl likewise at 40°C. and the two solutions are thoroughly mixed and kept at 40°C. for 3 minutes. The hemoglobin solution is now chocolate brown instead of deep red. To this solution is added a mixture of 3 cc. $\frac{N}{5}$ NaOH and 2 cc. $\frac{M}{5}$ KH_2PO_4 warmed to 40°C. The pH is now about 6.8, close to the isoelectric point of hemoglobin and in the region of minimum solubility of denatured hemoglobin. The flask is well shaken so as to facilitate subsequent filtration. The filtrate is clear and practically colorless. The nitrogen content of the filtrate is somewhat less than 0.05 mg. per cc., an amount that is just measurable. This experiment is carried out at 40°C. to facilitate filtration. When done at room temperature the filtrate is cloudy; at 40°C. it is clear. 40°C. is far below the coagulation temperature of hemoglobin in water, which is between 70° and 80°C.

To see whether native hemoglobin is carried down by denatured protein, the denatured protein is precipitated from a known mixture of native and denatured hemoglobin and the native protein estimated in the filtrate. The known mixture is prepared by adding a known amount of native hemoglobin to a known amount of denatured protein dissolved in acid. The pH and temperature must be such that the native protein is not denatured during the time of the experiment. Under these conditions no evidence of carrying down of native protein has been found. What would happen under conditions under which rapid denaturation takes place is not open to direct experiment.

To 10 cc. of the 10.85 per cent hemoglobin solution are added 20 cc. $\frac{N}{5}$ HCl and the mixture allowed to stand for 3 minutes. 20 cc. $\frac{N}{5}$ NaOH are added. The suspension is centrifuged, the supernatant fluid rejected, and water added. It is shaken and centrifuged again. To the suspension of denatured hemoglobin 3 cc. $\frac{N}{5}$ HCl are added. This dissolves most of the protein. The suspension is centrifuged to remove the undissolved portion. The volume of solution is now about 10 cc. and contains about 4.2 per cent protein. 0.5 cc. of 13.8 per cent of carbon monoxide hemoglobin is mixed with 4 cc. $\frac{M}{5}$ KH_2PO_4 and warmed to 40°C. Carbon monoxide hemoglobin is used in this experiment because it is more stable in acid than is either methemoglobin or oxyhemoglobin. This HbCO solution is added to 2 cc. of the solution of denatured hemoglobin in HCl. Immediately

afterward 2.6 cc. $\frac{N}{5}$ NaOH warmed to 40°C. are added. The mixture is well shaken and then centrifuged. The supernatant fluid has the color of HbCO and its concentration can be estimated colorimetrically. Within the error of the experiment (about 3 per cent) all of the HbCO added is recovered, that is to say, no native protein is brought down by the coagulum.

It is conceivable that methemoglobin or free native globin might behave differently from HbCO, so experiments have also been done with these proteins.

A solution of horse methemoglobin is prepared by adding potassium ferricyanide to an oxyhemoglobin solution until the absorption bands of oxyhemoglobin can no longer be seen. This solution is dialyzed against distilled water to free it from ferricyanide. After dialysis the protein concentration is 12 per cent and the specific conductivity is 1.4×10^{-4} reciprocal ohms. The methemoglobin is diluted to 5 per cent with distilled water. To 5 cc. methemoglobin are added 1 cc. $\frac{N}{10}$ HCl and 0.5 cc. $\frac{M}{2}$ NaCl. The solution is heated in boiling water for 3½ minutes and then cooled at 0°C. It is added to 5 cc. $\frac{M}{5}$ phosphate buffer (at 0°C.) of pH 6.8. The mixture is filtered and to the hazy filtrate half its volume of saturated $(NH_4)_2SO_4$ is added. This mixture is filtered, and the filtrate is now clear. This nearly colorless filtrate contains less than 1 per cent of the protein present before coagulation. The rest of the protein has been apparently coagulated. To see whether any methemoglobin is carried down by the coagulum the experiment is repeated but just before adding the buffer 1 cc. of 5 per cent methemoglobin is added. The final filtrate is now colored and its color is compared with that of a standard containing 1 cc. of 5 per cent methemoglobin and 17.25 cc. of the nearly colorless filtrate from the original coagulum. These two solutions are indistinguishable colorimetrically, indicating that no native protein is carried down by the coagulum.

If, instead of precipitating the denatured protein at its isoelectric point, it is precipitated from acid solution by ammonium sulfate it can be shown that no native protein is carried down by the coagulum. All of these experiments can be done, using slightly different quantities, with oxyhemoglobin. They can also be done using so-called native globin and denatured globin or hemoglobin. The result is always the same.

(*2*) *Heat.* When the coagulum is obtained simply by heating the

native protein, the filtrate contains practically no nitrogen. The record of an experiment is as follows:

To 7 cc. of a 10.85 per cent hemoglobin solution are added 10 cc. of water. The solution (in a test tube) is plunged into boiling water for 3½ minutes and then cooled in cold water. To the coagulum 6 cc. of a pH 6.8 phosphate buffer are added and mixed thoroughly with the protein. The suspension is filtered and the nitrogen content of the filtrate estimated by a micro-Kjeldahl method. Each cc. contains 0.065 mg. of nitrogen. If this were due to hemoglobin it would represent a solution of only about 0.035 per cent. This is negligible compared with the amount of soluble protein obtained from the coagulum.

In heat coagulation there is additional evidence that the coagulum contains no native protein and that the soluble protein does not come from such an impurity in the coagulum. Heat denaturation follows the course of a unimolecular reaction at all temperatures at which it can be measured (5). In experiments on the kinetics of this reaction the concentration of the reactant, native hemoglobin, is measured by filtering off the coagulum at any given time and estimating the hemoglobin concentration in the filtrate. To harmonize these facts with the assumption that native protein is carried down one must further assume that every molecule of denatured protein carried down a given and constant number of molecules of native protein.

(*3*) *Heat in Acid Solution.* The completeness of denaturation in this case can be tested not only by the observation of complete insolubility but also by a comparison of the yields of soluble protein obtained from hemoglobin denatured at two different temperatures. The denaturation of hemoglobin has a high temperature coefficient. If hemoglobin heated for 3½ minutes at 80°C. still contains some undenatured protein, then hemoglobin heated at 100°C. should contain at least a hundred times less. Did the soluble, apparently native hemoglobin obtained from the heat denatured hemoglobin come from undenatured protein, then one would expect to get much less of this soluble protein from the hemoglobin heated at 100°C. than from the hemoglobin heated at 80°C. Actually, as will be shown in the following paper, the yield is the same, about 30 per cent in both cases.

The preparation of the coagulated protein is carried out as follows:

To 5 cc. of a 14 per cent hemoglobin solution are added 7.25 cc. H_2O and 4.75 cc. $\frac{N}{5}$ HCl (just enough to keep the denatured hemoglobin in solution). The

solution is heated at 80°C. for 3½ minutes, and while still hot a mixture of 5 cc. $\frac{M}{5}$ KH_2PO_4 and 7.25 cc. $\frac{N}{5}$ NaOH is added to it and mixed at 80°C. The filtrate from this coagulum is clear and colorless. When it is heated to over 90°C. it shows practically no opalescence, indicating that coagulation is complete. Similarly the hemoglobin heated at 100°C. is completely precipitated on neutralization.

(*4*) *Urea.* We find that concentrated solutions of urea denature proteins and keep the denatured proteins in solution. If hemoglobin is allowed to stand in urea solution, then on removal of the urea by dialysis or on dilution of the solution the protein precipitates completely. Furthermore, as will be shown in a later paper, denaturation by urea can be followed by viscosity measurements. As denaturation proceeds, the solution becomes more viscous. Finally, when all the protein has been converted into a form which is insoluble in water when the urea is removed, the viscosity remains constant at a high level. Were denaturation as tested for by solubility incomplete one would expect a further change of viscosity with time.

The urea coagulum is prepared as follows:

200 grams of urea are dissolved in 250 cc. of a 14 per cent hemoglobin solution and allowed to stand for 48 hours at room temperature. The solution is added slowly and with stirring to about 4 liters of water. A flocculent precipitate appears. This is thoroughly washed with water by decantation. The coagulum is concentrated by centrifuging at low speed.

II. Hemoglobin as a Typical Coagulable Protein

In order that experiments with completely denatured hemoglobin may be of general significance in the study of coagulation it must be made clear that what we have called the denaturation of hemoglobin is the counterpart of the denaturation of other proteins, not a peculiarity of hemoglobin. We shall accordingly show in detail that all the procedures such as heating which convert egg albumin into an insoluble denatured form likewise convert hemoglobin into an insoluble form, and that the insoluble hemoglobin has besides insolubility in water the other characteristics of denatured protein. In making this comparison it has been necessary in some cases to provide the data for hemoglobin. On the other hand, where experiments on the denaturation of hemoglobin have led to the discovery of new phenomena,

these same phenomena have been found to exist in the denaturation of other proteins.

Hitherto in the investigation of some hemoglobin derivatives attention has been focussed on the non-protein part of the molecule and the protein part has been ignored. In such studies a denatured form of hemoglobin has been almost unknown. We now know (2) however, that the preparation of some hemoglobin derivatives, such as hemochromogen, involves the denaturation of the protein.

(*1*) *Heat.* Hemoglobin like egg albumin can be coagulated by heat. In both cases the denaturation proceeds as a monomolecular reaction with the striking and characteristic temperature coefficient of over 600 for 10° (9). The rate of most chemical reactions is increased from 2 to 4 times for a rise in temperature of 10°.

At first sight it might seem that the temperature coefficient of the denaturation of hemoglobin is different from that of other proteins, for although the coefficient for methemoglobin is over 600, for oxyhemoglobin it is only 13.8 and for carbon monoxide hemoglobin 5.2 (9). But when oxy or carbon monoxide hemoglobin is heated two entirely different reactions take place, the hemoglobin is converted into methemoglobin (that is, the iron atoms of the native protein are oxidized) and the protein is denatured. In order, therefore, to study heat denaturation alone apart from methemoglobin formation, it is necessary to start with methemoglobin.

Both dry hemoglobin and dry egg albumin can be heated to 100° without the protein losing its solubility in water.

(*2*) *Acid and Alkali.* If acid or alkali is added to either hemoglobin or egg albumin and the solution is subsequently brought to the isoelectric point of the protein, the protein is precipitated. Acid and alkali thus denature both hemoglobin and egg albumin.

(*3*) *Alcohol* coagulates both hemoglobin and egg albumin.

(*4*) *Ultra-violet light* coagulates both hemoglobin and egg albumin (8).

(*5*) *Shaking.* It is known (11) that egg albumin can be completely coagulated by shaking. We have found that the same holds true for hemoglobin.* As in the case of heat coagulation, it is desirable to start the experiments with methemoglobin.

* Since this section was written Wu and Ling (16) have published an account of the coagulation of hemoglobin by shaking.

If a solution of methemoglobin (protected from bacterial reduction by a little toluol) is shaken, coagulated hemoglobin gradually appears. If more toluol is added and an emulsion with a large surface is formed, much more protein is coagulated. For example, a 10 per cent solution of salt-free methemoglobin is shaken for 16 hours with an equal volume of toluol. All the hemoglobin is coagulated. The supernatant fluid after centrifugation of the precipitate is clear and colorless. As a control experiment a similar methemoglobin solution containing only enough toluol to saturate the solution is allowed to stand 16 hours. Very little coagulum is formed. This makes it improbable that toluol itself coagulates hemoglobin.

On long standing methemoglobin gradually coagulates, as egg albumin does, even without shaking. It appears then that both hemoglobin and egg albumin coagulate at surfaces and that shaking merely accentuates this property.

(*6*) *Thiocyanate, Iodide, and Salicylate.* We have found that dilute solutions of these salts denature hemoglobin. Von Fürth (6) found that 5 per cent solutions of the same salts coagulate myogen. So thiocyanate, iodide, and salicylate probably denature proteins in general.

The experiments are carried out as follows:

1/10 cc. of salt-free methemoglobin (about a 10 per cent solution) is added to 5 cc. of a solution containing KI, KCNS or Na salicylate. The salts are present in a concentration of 0.6 M. In one series these salts are in an acetate solution buffered at pH 5.2 and in another series they are in a borate solution of pH 9.2. The acid tubes all contain precipitates after 12 hours. A pigment in the alkaline tubes is still in solution and no precipitate is detectable.

A similarity between this method of denaturation and others may be tested when the alkaline solution containing any one of these three salts is reduced with sodium hydrosulfite; it then has the absorption spectrum of hemochromogen, the substance formed when methemoglobin is reduced and denatured—whether by heat, acids, alkalies, alcohol, ultra-violet light or shaking. It is therefore probable that methemoglobin solutions at pH 9.2 are also denatured by the KI, KCNS, and Na salicylate.

These solutions (without addition of sodium hydrosulfite) are now

placed in collodion tubes and dialyzed against distilled water as the outer liquid. As dialysis proceeds, a brown precipitate appears in the collodion tubes. This precipitate has the same properties as the coagulum formed from methemoglobin by the action of the various agents we have studied. We therefore conclude that methemoglobin has been denatured by 0.6 M solutions of these salts.*

(7) *Urea.* As described in the first part of this paper, concentrated urea solutions denature hemoglobin and keep denatured hemoglobin in solution. When the urea solution is diluted, the protein precipitates completely and can be washed free of urea. Apart from the precipitation of the protein on dilution of the urea, there is spectroscopic evidence that the hemoglobin has been denatured. If the reducer, $Na_2S_2O_4$, is added, the spectrum of hemochromogen, that is denatured hemoglobin, can be observed.

We find that urea has the same effect on egg and serum albumin that it has on hemoglobin. A concentrated urea solution is added to some egg albumin and the solution is allowed to stand for a day. When a few drops are added to about 15 cc. of water buffered at a pH of 4.7 (isoelectric point of egg albumin) a voluminous protein precipitate occurs. The protein redissolves when urea crystals are dissolved in the water. Apparently urea can denature egg albumin and likewise dissolve the denatured protein.

If the same experiment (with urea) is repeated with serum albumin it is found that no precipitate appears when several times as much water is added as in the preceding experiment. The solution is placed in a collodion tube and dialyzed overnight against running distilled water. Next morning the protein is found precipitated at the bottom of the tube. It would seem as if urea denatures serum albumin and also dissolves denatured serum albumin, but a less concentrated urea solution is needed to prevent the precipitation of denatured serum albumin than is needed in the case of denatured hemoglobin or egg albumin.

It has been shown that heat, acid, alkali, alcohol, ultra-violet light, KCNS, KI, Na salicylate and urea all convert hemoglobin as they do egg albumin into an insoluble form, and that the kinetics of dena-

* Other experiments show that much more dilute solutions of these salts suffice to denature hemoglobin.

turation is the same in the cases of hemoglobin and egg albumin. In order to complete the evidence that hemoglobin is a typical coagulable protein, it remains to show that the insoluble protein formed from hemoglobin by heat, etc., has in addition to insolubility in water all the other characteristic properties of denatured protein.

(*1*) *Solubility.* Both denatured hemoglobin and denatured egg albumin after being precipitated at their isoelectric points can be dissolved again in acid or alkali. Even at its isoelectric point denatured hemoglobin can be dissolved not only in concentrated urea solution, as has already been pointed out, but also in concentrated solutions of KCNS, KI, and Na salicylate. A urea solution not concentrated enough to dissolve isoelectric coagulated hemoglobin, may still suffice to dissolve the coagulum if the solution is slightly acid or alkaline, so slightly acid or alkaline that without the urea, very little of the protein would dissolve. Denatured egg and serum albumins are likewise soluble in solutions of urea and of the salts mentioned. As Spiro (14) noticed, serum albumin can be heated in saturated urea solution without any visible precipitate being formed.

(*2*) *Viscosity.* We have found that when either hemoglobin or egg albumin is denatured there is an increase in viscosity characteristic of denaturation. This change is of a greater order of magnitude than the changes associated with the ionization of the soluble proteins, which are the concern of most investigation of the viscosity of protein solutions. Our experiments on the viscosity of solutions of denatured proteins will be published in a separate paper.

(*3*) *Species Specificity.* When proteins are denatured, their species specificity, as determined immunologically, is decreased. No similar immunological experiments have been done with denatured hemoglobins. But, as we have shown in previous papers (1, 2) it is impossible to distinguish the various denatured hemoglobins by spectroscopic or gas affinity measurements, although by these same methods it is easy to distinguish native hemoglobins of even the most closely related species.

SUMMARY

As a preliminary to the study of the reversal of the coagulation of hemoglobin several methods are described for the preparation of

completely denatured and coagulated hemoglobin and the evidence is given that hemoglobin is a typical coagulable protein.

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PROTEIN COAGULATION AND ITS REVERSAL

The Reversal of the Coagulation of Hemoglobin

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The coagulation of hemoglobin takes place in two steps (7, 5, 6). The first step is denaturation, a chemical change in the protein which makes it insoluble around its isoelectric point. The second step is merely the precipitation of the insoluble denatured protein. It has hitherto been believed that denaturation is irreversible.

The preceding paper (4) has already described the preparation of completely coagulated hemoglobin and has given the evidence that the denaturation and coagulation of hemoglobin are in all known respects like the denaturation and coagulation of other proteins such as egg albumin. The first part of this paper describes the preparation from completely coagulated hemoglobin of soluble, apparently native hemoglobin. The second part gives the evidence that this soluble protein is the same as the original native hemoglobin which had never been modified. From these experiments the conclusion is drawn that the coagulation of hemoglobin is reversible. Since hemoglobin is a typical coagulable protein, if the coagulation of hemoglobin is reversible then the coagulation of proteins in general is probably reversible. The coagulation of other proteins, in particular globin and the tissue proteins, and the biological significance of coagulation will be discussed in later papers.

Part I. The Preparation of Soluble Hemoglobin from Coagulated Hemoglobin

If an acid or alkaline solution of completely coagulated hemoglobin is neutralized all the protein is precipitated. If, however, before com-

plete neutralization the solution of denatured hemoglobin is allowed to stand in slightly alkaline solution under conditions to be described, then on complete neutralization only a part of the protein is precipitated. The remaining soluble part is the apparently reversed hemoglobin. How much of this soluble apparently reversed hemoglobin is obtained depends, other factors being constant, on the state of the prosthetic group, heme, and on the species of the hemoglobin.

In the case of the ox hemoglobin, if the heme is in the oxidized state, very little soluble hemoglobin is obtained from the coagulum. If a little of the reducer, sodium hydrosulfite, is added, much more is obtained. And if cyanide is added still more is obtained.

In the case of horse hemoglobin, practically no soluble protein is obtained from the coagulum whether the heme is either oxidized or reduced. When cyanide is used, however, about 30 per cent of the denatured protein yields soluble crystalline apparently native horse hemoglobin. Because it can be crystallized most of the experiments have been done with horse hemoglobin.

The results with ox hemoglobin show that cyanide is not essential for the apparent reversal of coagulation. And even though cyanide appears to be necessary for an adequate yield with horse hemoglobin, neither the initial soluble nor the final soluble product is combined with cyanide. So the question of the function of the cyanide is separate from the question of whether denaturation is reversible.

The experiments with horse hemoglobin show clearly that the mere fact that denaturation cannot be reversed under a given set of conditions does not mean that denaturation cannot be apparently reversed under other more suitable conditions.

It is to be expected that the state of the prosthetic group and the species of the hemoglobin should influence the ease of the apparent reversal of denaturation because these same factors are known to influence the ease of denaturation itself. Some hemoglobins are more readily denatured than others and methemoglobin is more readily denatured than oxy or carbon monoxide hemoglobin. That the heme part of the hemoglobin molecule should modify its protein properties is not surprising. We have already shown (2) that the globin part of the molecule modifies greatly the properties of the non-protein heme part, in particular its reactions with oxygen.

The mechanism of the effect on apparent reversal of the reducer, $Na_2S_2O_4$ and of cyanide is not yet understood. It is not even certain that $Na_2S_2O_4$ and CN act solely on the non-protein part of hemoglobin. It might be desirable, nevertheless, to recall the reactions of cyanide with the various hemoglobin derivatives. Cyanide combines with both oxidized and reduced heme when the heme is joined to denatured globin but only with oxidized heme when the heme is joined to native globin. In other words, cyanide combines with hematin, hemochromogen and methemoglobin but not with oxy, carbon monoxide or reduced hemoglobin.

One step in the procedure for obtaining apparently reversed horse hemoglobin consists in dissolving the coagulated protein in a slightly alkaline solution containing cyanide. It is desirable to buffer the solution in order to protect the protein which is sensitive to alkali. The dissociation constant of hydrocyanic acid is 72×10^{-10}, about the same as that of boric acid. Mixtures of NaCN and HCl may accordingly be used as buffers in the region around pH 9.14. Cyanide, then, is used in these experiments for two entirely different purposes, to facilitate apparent reversal by reacting with denatured hemoglobin and as part of a buffer mixture to maintain a suitable hydrogen ion concentration.

Technique for Apparent Reversal of Coagulation

Heat Coagulated Ox Hemoglobin. A hemoglobin solution is prepared from ox blood by laking the washed corpuscles with ether. To 1 cc. of the hemoglobin solution are added 8 cc. of water and 1 cc. $\frac{N}{5}$ HCl; the solution is then heated at 80°C. for 3½ minutes. While at 80°C. a mixture of 3 cc. $\frac{M}{5}$ KH_2PO_4 and 2½ cc. $\frac{N}{5}$ NaOH is added so as to bring the pH to 6.8. The suspension is cooled and then filtered. The filtrate is clear and colorless. When boiled it turns only faintly opalescent indicating that practically all the hemoglobin has been coagulated.

In order to obtain the apparent reversal of coagulation the denaturation experiment is repeated but instead of adding the phosphate buffer the solution is allowed to cool and 10 cc. of $\frac{N}{25}$ NaOH are added making it faintly alkaline. A little $Na_2S_2O_4$ is added and the mixture allowed to stand for 2 to 3 minutes. With a spectroscope the absorption bands of both hemochromogen and reduced hemo-

globin are visible. The suspension is filtered and the filtrate gently shaken in the air to oxygenate. The absorption bands of the soluble, apparently native HbO_2 can be distinctly seen. If the heating is at the boiling point (20° higher) substantially the same result is obtained. The only difference is that now the absorption bands of the soluble oxyhemoglobin are a little fainter. If this soluble HbO_2 were merely some native protein that had not been coagulated, hundreds of times as much HbO_2 would be obtained from the solution heated at 80°C. as from that at 100°C.

In the case of horse hemoglobin the denatured protein is first dissolved in acid. Alkali causes secondary changes in the protein beyond mere denaturation. The acid solution is brought to about pH 9 or slightly more alkaline with a KCN-HCl buffer. The solution is then neutralized and the resulting precipitate filtered off. The filtrate is saturated with CO and reduced with $Na_2S_2O_4$. Finally ammonium sulfate is added. The amorphous precipitate first found settles to the bottom. The pigment in the clear supernatant fluid gradually separates out completely in the form of crystalline apparently native carbon monoxide hemoglobin. Carbon monoxide is used because carbon monoxide hemoglobin is more stable than oxyhemoglobin. Oxyhemoglobin together with methemoglobin is obtained if the carbon monoxide is not used and the reducer is oxidized.

The cyanide buffer solution is made by adding to 1 gm. of KCN dissolved in 4.16 cc. water 23.13 $\frac{N}{5}$ HCl. The solution is blue to thymol blue.

Preparation of Hemoglobin. Red corpuscles washed four times with isotonic salt solution are shaken up with an equal volume of water and a fifth the total volume of toluol (8). After the solution has stood overnight in the cold, the toluol and stromata are removed by filtration or centrifugation.

Heat Coagulated Horse Hemoglobin. A tube containing 7 cc. of 10.85 per cent of horse hemoglobin and 10 cc. of water is placed in boiling water for 3½ minutes. The temperature rises to 80°C. in about half a minute and then gradually goes up to a little over 90°C. The contents are not agitated, thus facilitating subsequent solution of the coagulum. The tube is placed in cold water for a few minutes. 4 cc. $\frac{N}{5}$ HCl are poured in (down the side of the tube) without stirring. The contents of the tube are mixed gently as the coagulum dissolves and then poured into a 50 cc. flask. 4 cc. $\frac{N}{5}$ NaOH and 2.5 cc. of the cyanide buffer solution are mixed and added to the flask making its contents faintly alkaline. A brownish red precipitate is observed. The mixture is allowed to stand for 2 to 6 hours. Most of the precipitate gradually redissolves. 5 cc. $\frac{M}{5}$ KH_2PO_4 are added to bring the mixture nearer the neutral point, to a pH of about 7 to 7.4. A brownish red precipitate is deposited. The suspension is filtered or centrifuged and a deep red solution obtained. Some carbon monoxide is bubbled

through this and immediately a knife point of solid $Na_2S_2O_4$ is added to reduce the solution. The flask is stoppered, gently shaken and then allowed to stand in the dark for about a half hour. To the solution is added about one and one-half times its volume of saturated $(NH_4)_2\ SO_4$. The mixture is allowed to stand in the dark for 24 to 48 hours. A flocculent, amorphous precipitate gradually settles out and is discarded. The clear, supernatant fluid is poured into a tall vessel. Through this fluid a little carbon monoxide is bubbled. The vessel is stoppered and allowed to stand in the dark. In the course of a few days large crystals gradually appear on the walls and bottom of the flask. At the end of a month, if not before, practically all of the color has left the solution and there is a large deposit of crystals. There is practically no amorphous matter mixed with the crystals. Some of them are so large that their form can be observed with the unaided eye. They can be washed with a little of the $(NH_4)_2SO_4$ solution. These crystals constitute the soluble apparently reversed protein.

Horse Hemoglobin Denatured by Acid. 6.5 cc. of the 10.85 per cent horse hemoglobin solution are mixed with 10 cc. $\frac{N}{5}$ HCl and the mixture kept at 40°C. for 3 minutes. After cooling a mixture of 2.5 cc. of the cyanide solution used above to dissolve the heat coagulum and 10 cc. $\frac{N}{5}$ NaOH are added to the denatured hemoglobin. From this point the procedure is exactly the same as that described above. In some of our experiments before obtaining an alkaline solution of denatured protein the acid solution was carefully neutralized, so that the denatured protein precipitated. This precipitate was thoroughly washed with distilled water not only until the washings were colorless, but even until they gave no test for chloride. The precipitate was then dissolved in alkali.

Horse Hemoglobin Denatured in Acid at Two Different Temperatures. The same yield of apparently reversed hemoglobin is obtained whether the protein is heated in acid at 80°C. or 100°C. The rate of denaturation of oxyhemoglobin is more than a hundred times faster at the higher than at the lower temperature. Did the soluble protein come from protein which had never been denatured then one ought to get much less soluble protein from the protein heated to 100°C. than from the protein heated to 80°C. Since the same yield is obtained in both cases the final soluble protein is probably not merely native protein which had never been denatured. Just enough acid is used to prevent visible precipitation. Boiling a neutral suspension of coagulated hemoglobin causes the protein to form hard clumps which are hard to dissolve. If the acid is neutralized immediately after the heating all the protein precipitates indicating complete denaturation.

To 5 cc. of 13.8 per cent hemoglobin are added 7.25 cc. water and 4.75 cc. $\frac{N}{5}$ HCl. The solution is heated for 3½ minutes. After cooling, a mixture of 4.75 $\frac{N}{5}$ NaOH and 2.5 cc. cyanide buffer is added. The rest of the procedure is as already described.

Hemoglobin Denatured by Urea. About 15 cc. of the suspension of protein denatured by urea prepared as described in the preceding paper are dissolved by the addition of 4 cc. $\frac{N}{5}$ HCl, and the procedure is then completed in the usual manner. The crystals obtained are less perfect than those obtained in the other cases, and the yield is smaller.

The Yield. The yield in the experiments described in this paper was determined colorimetrically, using carbon monoxide hemoglobin in ammonium sulfate solution as a standard. The solutions were taken for estimation after the addition of ammonium sulfate, after the separation of the amorphous precipitate, but before the separation of crystals. A part of the solution was always kept to make sure that all the pigment would eventually separate out in crystalline form. The solutions contained no pigment other than carbon monoxide hemoglobin which could be detected spectroscopically and they matched in color the standard solutions.

In the case of the horse hemoglobin denatured by heating, by acid or by heating in acid, the yield of apparently native carbon monoxide hemoglobin is about 30 per cent of the total original hemoglobin. Some of the remaining 70 per cent was of course lost in purely mechanical ways.

We are now investigating the factors influencing the yield and the yields obtained when one starts with the "reversed" and the "non-reversed" fractions.

Part II. Comparison of Apparently Reversed and Native Horse Hemoglobin

The chemical changes in the apparent reversal of denaturation and coagulation are as obscure as the changes in denaturation itself. In order, however, to prove the possibility of reversal it is not essential to investigate or understand the mechanism of any intermediate reactions. It is necessary to examine only the initial and final prod-

ucts, to show that one starts with coagulated protein and that one finally obtains from it native protein.

The preceding paper has given the evidence that the initial product was completely denatured. It remains to examine whether the soluble apparently reversed horse hemoglobin finally obtained from completely coagulated horse hemoglobin has not only all the general characteristics of native hemoglobin but quantitatively the precise properties of native horse hemoglobin. The apparently reversed and the original native proteins have accordingly been compared qualitatively in respect to solubility,[1] and quantitatively in respect to coagulation, crystal form, color, spectrum, reactions with oxygen and carbon monoxide, and species specificity. No differences have been found.

The advantages of using hemoglobin, a conjugated protein, are now apparent, for due to the union of protein to the iron-pyrrol complex, the molecule possesses in addition to all the ordinary protein properties, others open to exact measurement that simple proteins do not possess. Since practically all of the properties of the iron-pyrrol complex in hemoglobin are affected by the state of the globin, changes in the protein are reflected in the clearly defined behavior of the non-protein part of the molecule.

It is possible that there are differences between the apparently reversed and the original native proteins which have not been detected. A deeper knowledge than we now possess of denaturation and reactions resulting in apparent reversal may make such differences significant. For the present, however, the most reasonable conclusion from the experiments described in this paper is that the denaturation and coagulation of hemoglobin, and hence probably of the other coagulable proteins, are indeed reversible.

The Crystals. A denatured protein has never been crystallized, but the soluble hemoglobin obtained from coagulated hemoglobin is readily crystallizable. In the present experiments the protein is crystallized by salting out slowly with $(NH_4)_2SO_4$. The less concentrated the hemoglobin solution the more salt is needed; on the other hand, the less salt used the slower the crystallization, and consequently

[1] Quantitative solubility measurements are now being carried out.

the larger and more perfectly formed the crystals. The salt concentration suitable for crystallizing apparently reversed protein is the same as for crystallizing native carbon monoxide hemoglobin from a protein solution of the same concentration. The crystals obtained (Fig. 1) are of the same crystal form and habit as the crystals of native horse HbCO. Most of the further examination of the apparently reversed protein is of a solution of these crystals.

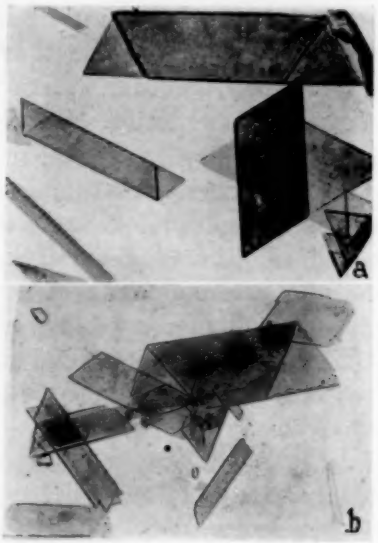

FIG. 1 *a* and *b*. (*a*) crystals of carbon monoxide hemoglobin; (*b*) crystals of "reversed" carbon monoxide hemoglobin.

Since the apparently reversed hemoglobin can be crystallized and by a procedure not described in this paper crystallized completely in a few minutes, the apparently reversed hemoglobin is a definite pure substance and not merely a vague disintegration product of denatured hemoglobin.

Solubility. Denatured hemoglobin is practically insoluble in water at its isoelectric point, about pH 6.8. It does not even color the water. The crystals, however, are readily soluble in a pH 6.8 phosphate buffer. A deep red solution containing 5 per cent of apparently native HbCO is easily prepared.

Coagulation. The solution of apparently reversed protein can be coagulated by heat. The temperature of coagulation is a highly characteristic constant of a protein. It is, however, affected by salt. If apparently reversed protein dissolved in a $(NH_4)_2SO_4$ solution is compared with a solution of native HbCO of the same concentration in the same salt solution by placing the tubes containing them side by side in boiling water, coagulation occurs at the same instant in both solutions. The temperature of coagulation of the apparently reversed protein is therefore the same as that of the native protein from which it is prepared. The apparently reversed protein can be denatured by acid, alkali, etc.

Color. The color of the apparently reversed protein matches that of ordinary horse HbCO, when they are compared in the colorimeter. It has already been stated that when $(NH_4)_2SO_4$ is added to the solution of apparently reversed protein an amorphous precipitate settles out before crystallization occurs. This precipitate is discarded. If, however, the color of the solution is examined before the removal of this substance, the color is distinctly different from that of uncoagulated protein, showing that with the colorimeter it is possible to detect a small amount of foreign pigment. The apparent coagulation temperature of this unpurified preparation may be more than 20° lower than that of original native protein.

Absorption Bands. The absorption spectrum of the apparently reversed protein is indistinguishable from that of native hemoglobin. With the Hartridge reversion spectroscope the positions of the absorption bands can be measured to about 2 Å. u., but we have been unable to detect any difference in the patterns of the absorption spectra or the positions of the absorption bands of the HbO_2, HbCO, methemoglobin, or sulfhemoglobin obtained from the apparently reversed and the original native protein.

Combination with Oxygen. Denatured hemoglobin does not form that loose combination with oxygen so characteristic of hemoglobin. The apparently reversed hemoglobin combines loosely with oxygen and can be oxygenated and reduced repeatedly.

Gas Affinities. Hemoglobin combines loosely with both oxygen and carbon monoxide. If hemoglobin is exposed to a mixture of the two gases at equilibrium, part of the pigment is combined with oxygen

and part with carbon monoxide. The reaction obeys the mass law equation.

$$\frac{[HbCO]\,[O_2]}{[HbO_2]\,[CO]} = K$$

K, the equilibrium constant, is a measure of the relative affinities of hemoglobin for the two gases and is different for each species of hemoglobin. Barcroft, Oinuma and the writers (1) showed that K can be predicted from purely spectroscopic measurements, being a function of the number of Ångström units separating the α bands of oxy and carbon monoxide hemoglobins. Since these bands are in the same positions in the apparently reversed hemoglobin as in ordinary native hemoglobin, one would expect the K's to be the same in both cases, too. This we have found to be the fact.[2] The technique has already been described (1). To make sure that equilibrium was reached, the equilibrium was approached from both sides.

In developing the method for determining K it was found possible to estimate the relative quantities of HbO_2 and HbCO in a mixture of both by measuring the position of the α absorption band of the mixture. The position of this band is a function of the composition of the mixture. This function is exactly the same for HbO_2 and HbCO of apparently reversed hemoglobin as it is for original hemoglobin. In one more way, therefore, the spectroscopic properties of apparently reversed hemoglobin are quantitatively the same as those of native hemoglobin.

Species Characteristics. By means of crystal form and by spectroscopic and gas affinity measurements (1) the hemoglobins of different species are easily distinguished. The soluble, apparently native hemoglobin prepared from coagulated horse hemoglobin even by these sensitive tests cannot be distinguished not only from native hemoglobin in general but from native horse hemoglobin in particular. This is especially interesting because, as we have already shown (2, 3) denatured hemoglobins of various species cannot be distinguished from each other by spectroscopic or gas affinity measurements. After apparent reversal of coagulation the species characteristics once more become observable.

[2] This experiment was performed only once, though in duplicate, so a repetition would be desirable.

SUMMARY

1. The preparation from completely coagulated hemoglobin of crystalline soluble hemoglobin is described.

2. This soluble hemoglobin by all the tests tried has been indistinguishable from normal native hemoglobin which has never been coagulated.

3. The coagulation of hemoglobin is probably reversible.

4. Since hemoglobin is a typical coagulable protein, protein coagulation in general is probably reversible.

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THE PREPARATION OF RELATIVELY PURE BACTERIOPHAGE

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Studies on the nature of the bacteriophage are usually carried out with lysates which contain, in addition to the lytic principle itself, the nutrient constituents of the broth and substances set free from the lysed bacteria. While such preparations are suitable for many types of investigation, there are certain problems, *e.g.*, those dealing with the physical or chemical properties of the bacteriophage, which require the use of relatively pure phage.

That the composition of the medium in which bacteriophage is suspended exerts considerable influence on the physical state of the lytic agent was brought out by recent work of the authors (1), who showed that purified bacteriophage free from bacterial proteins not only has a smaller particle size than non-purified phage but also will readily unite with proteins or protein derivatives to form the larger particles found in ordinary lysates. This point, that the lytic agent is commonly associated with colloidal aggregates has been repeatedly stressed by Bronfenbrenner (2) and it appears quite logical that physical-chemical experiments dealing with the phage itself should either be performed with purified bacteriophage free from the influence of a complex suspending medium such as nutrient broth, or else that the influence of the menstruum must be taken into account.

Arnold and Weiss (3) devised a means of preparing bacteriophage free from bacterial proteins based upon the diffusion of lytic corpuscles from a lysogenic culture seeded on nutrient agar into a bottom layer of plain agar. Several extractions are made from the latter stratum and one finally obtains a clear uncolored phage suspension which gives a slight positive reaction with the usual protein reagents. This may be further purified by precipitating the small residual protein

fraction with 14 per cent sodium sulfate. Such preparations are free from antigenic bacterial proteins as evidenced by their failure to produce antibodies other than antilysins when injected into animals.

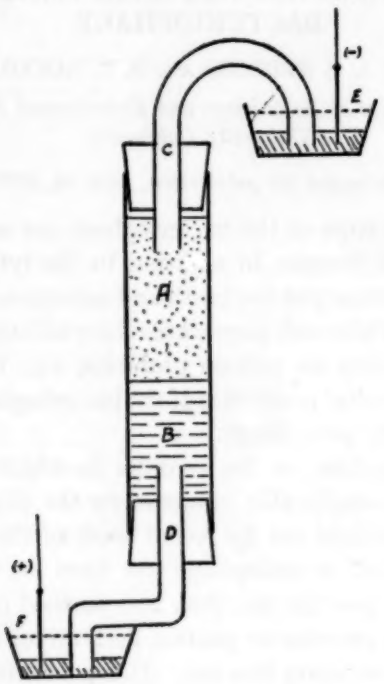

FIG. 1. Apparatus for preparation of relatively pure bacteriophage. *A*, bacteriophage suspension. *B*, gel of 0.5 per cent purified agar and 0.2 per cent C. P. NaCl in distilled water. *C* and *D*, glass bridges containing 3.0 per cent pure agar gel with 0.5 per cent C.P. NaCl. *E*, porcelain cup with crystals of $CuCl_2$ at bottom. Water to level of dotted line. Negative pole (Copper) of D.C. circuit dips into crystals. *F*, porcelain cup with crystals of NaCl at bottom. Water to level of dotted line. Silver foil attached to positive pole of D.C. circuit dips into crystals.

This procedure has the disadvantage, however, that during the numerous extractions required to remove the proteins much of the phage is removed as well and in our hands the method has not yielded

protein-free suspensions of high lytic titre. The most active preparation we were able to make following Arnold and Weiss' process was not effective beyond a dilution of 10^{-5}.* In addition the concentration of sodium sulfate in the final product is objectionable for certain types of work.

Investigations we had under way demanded the use of strongly lytic suspensions containing a minimum concentration of the other materials found in ordinary lysates. Since it is known that the bacteriophage carries a negative charge from pH 3.6 to pH 7.6 (Todd) (4) it seemed quite reasonable to attempt its concentration at the anode of some sort of cataphoresis apparatus, employing, if possible, a pH lower than the isoelectric ranges of the other constituents of the lysate in order to retain them at the cathode. After numerous experiments with various forms of apparatus the following procedure was developed and has proven entirely satisfactory.

Method of Preparing Relatively Pure Bacteriophage Suspensions

Pure agar is prepared according to the method of Dominikiewicz (5). To a 0.5 per cent suspension of the pure agar in distilled water 0.2 per cent C.P. NaCl is added and the mixture is sterilized by boiling or autoclaving.

The apparatus is explained in the accompanying diagram and requires no special mention except to note that convenient dimensions for the tube are 15 cm. by 2.5 cm. It is our practice to sterilize the assembled apparatus and to then fill the bridges with sterile purified 3 per cent agar containing 0.2 per cent sodium chloride. This is readily done by pipetting the agar into the inverted upper bridge and by resting the distal end of the lower bridge against a flat surface while it is being filled. When the agar is hardened the lower bridge and stopper are fitted in place. 20 cc. of the 0.5 per cent agar containing 0.2 per cent sodium chloride is poured into the cylinder observing sterile precautions. After the agar is hard 30–40 cc. of ordinary phage suspension is layered over it and the upper stopper placed in position.

A current of 100–125 volts and from 5–12 milliamperes is passed through the apparatus for 18 to 20 hours (this need not be continuous). The phage above the agar is best changed once or twice during the run. At the end of this period the broth is taken up by pipette, discarded, and the upper surface of the agar washed

* We have employed the technic recommended by d'Herelle (The bacteriophage and its behavior, Williams and Wilkins, 1926, 96) in all quantitative estimations of the lytic particles.

with several changes of sterile physiological saline solution. The lower bridge and stopper are removed and the agar allowed to slide out gradually. It is sliced into thin sections with a sterile spatula as it issues forth and is received in a sterile Petri dish, discarding the layer 0.5 cm. thick nearest the broth. The remainder is thoroughly macerated for 2–3 hours with 10–30 cc. of sterile water or saline solution, depending upon the type of suspension and the concentration desired. It is then freed from agar particles by filtration through a coarse Chamberland candle or by centrifuging.

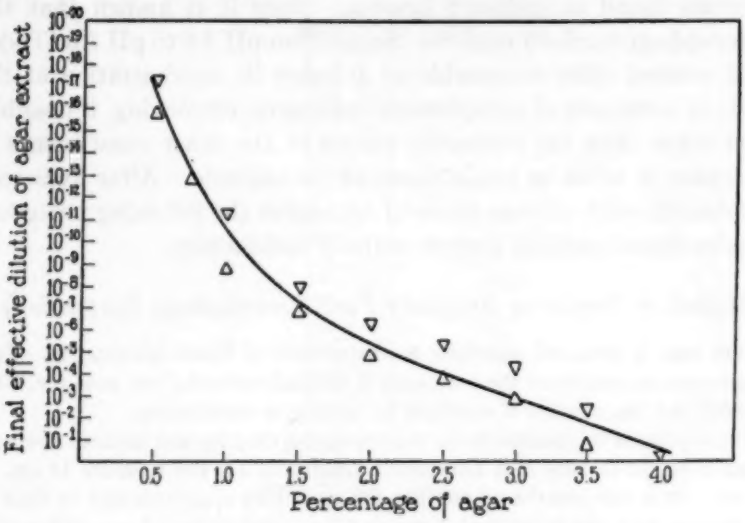

CHART 1. Effect of agar concentration on final phage content of extract. For each experiment: E.M.F. 100–102 V. 7–10 milliamperes. Current on 18 hours. 20 cc. agar of varying percentages from 0.5 per cent to 4 per cent all containing 0.2 per cent C.P. NaCl. Extractive: 20 cc. physiological saline. Anti-*coli* bacteriophage. Broth pH 7.4.

DISCUSSION

It was at first thought that the phage suspension used in the apparatus should be adjusted to a relatively low pH on the acid side of the isoelectric points of the proteins and their derivatives so that these substances would carry a positive charge and would therefore concentrate at the cathode. However, experiments run with ordinary Martin's broth suspensions (pH 7.0–7.4) constantly resulted in the

precipitation at the cathode of a dense coagulum giving the usual qualitative protein reactions. Furthermore, there was no migration of these compounds into the agar. Consequently, any such adjustment of pH was found unnecessary for our present purposes.

Early experiences indicated that the percentage of agar employed as well as the length of time during which a given E. M. F. is allowed to act markedly influenced the lytic titre of the extract. Two sets of experiments were accordingly undertaken to determine the optimal

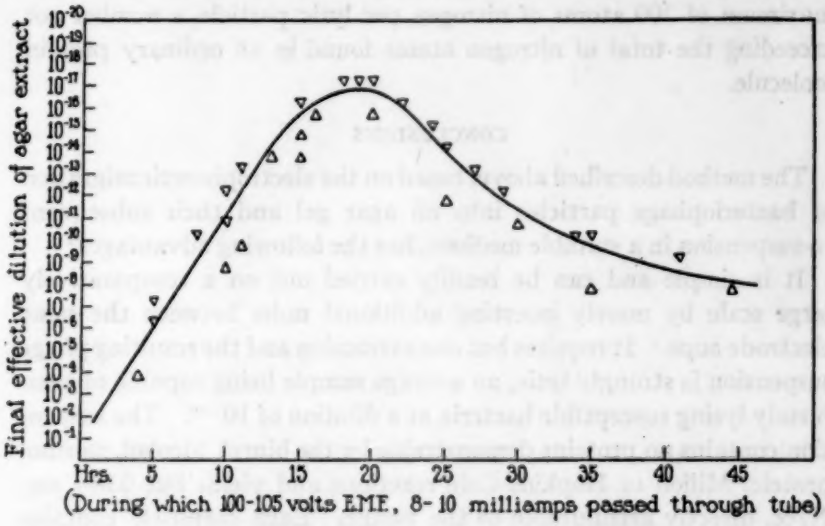
Final effective dilution of agar extract
Hrs. 5 10 15 20 25 30 35 40 45
(During which 100-105 volts E.M.F., 8-10 milliamps passed through tube)

CHART 2. Effect of varying time of current passage on final phage content of extract. For each experiment: E.M.F. 100–105 V. 8–10 milliamperes. 20 cc. 0.5 per cent agar with 0.2 per cent C.P. NaCl in distilled water. Extractive: 10 cc. physiological saline. Anti-*coli* bacteriophage. Broth pH 7.4.

conditions for preparing concentrated suspensions. Charts 1 and 2 present the results in graphic form, and indicate that a maximum concentration is obtained with a 0.5 per cent agar gel through which the current passes for 18–20 hours.

Failure to detect with ordinary qualitative reactions any traces of protein or protein-derivatives in the purified suspensions led us to determine the total nitrogen content per cc. The following figures are the averages of several analyses of each type of preparation:

(*a*) Non-purified anti-*coli* bacteriophage in Martin's broth: 0.548 mg. N/cc.
(22×10^8 corpuscles/cc.)
(*b*) Purified anti-*coli* bacteriophage in saline solution: 0.092 mg. N/cc.
(1×10^{10} corpuscles/cc.)
(*c*) Saline extract of purified agar without phage: 0.048 mg. N/cc.

Consequently, we may attribute to the phage itself (1×10^{10} corpuscles) only $0.092 - 0.048 = 0.044$ mg. N/cc. This calculates to a maximum of 200 atoms of nitrogen per lytic particle, a number not exceeding the total of nitrogen atoms found in an ordinary protein molecule.

CONCLUSIONS

The method described above, based on the electrophoretic migration of bacteriophage particles into an agar gel and their subsequent re-suspension in a suitable medium, has the following advantages:

It is simple and can be readily carried out on a comparatively large scale by merely inserting additional units between the same electrode cups. It requires but one extraction and the resulting phage suspension is strongly lytic, an average sample being capable of completely lysing susceptible bacteria at a dilution of 10^{-16}. The suspension contains no proteins demonstrable by the biuret, alcohol, xanthoproteic, Millon or Hopkins-Cole reactions and yields but 0.044 mg. N/cc. directly attributable to the phage. Each corpuscle contains no more nitrogen than a single molecule of protein.

In addition the method is applicable to determinations of the electric charge carried by biologically active substances of small dimensions, *e.g.*, phage, toxins, and perhaps some viruses. It offers as well a possible means of purification of these substances.

The purified bacteriophage obtained by such a procedure or similar ones is relatively unstable. Work now in progress indicates that it does not possess nearly the resistance to chemical agents, drying, etc., that non-purified phage displays.

It is suggested that experiments designed to test the therapeutic value of bacteriophage be conducted, when possible, with purified suspensions thereby avoiding any possibility of obscure non-specific reactions due to other constituents of the lysates.

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MEASUREMENTS OF THE METABOLISM OF TWO PROTOZOANS

By ROBERT EMERSON*

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(Accepted for publication, July 21, 1929)

Dr. J. A. Dawson of the Department of Zoology, Harvard University, has been kind enough to place at the writer's disposal relatively large quantities of two protozoans ("*Amoeba proteus*" according to Schaeffer, 1916; and *Blepharisma undulans*) in sufficiently pure condition to make possible some experiments on their metabolism. The results of these experiments, together with some observations on the red pigment of *Blepharisma* are recorded here. Dr. Dawson describes in a separate paper his methods of culturing the organisms.

I

Experiments on Amoeba proteus

The amebae obtained were always accompanied by large numbers of *Chilomonas*. In order to be sure that any metabolism found should refer to *Amoeba proteus* alone, the *Chilomonas* cells, which are much smaller in size, were washed out on a 200-mesh phosphor-bronze sieve, which retained all amebae. After two washings, a microscopical examination of the amebae showed them to be practically free of *Chilomonas*. The former were then concentrated by allowing them to settle in shallow dishes, and decanting. They were washed once or twice on the centrifuge to remove bacteria, and a suspension of them was made up and pipetted into the vessels of Barcroft-Warburg manometers.

Various suspending solutions were tried, similar in composition to Ringer, but much more dilute. None proved successful, however, and metabolism was demonstrable only in distilled water. The water used was commercial distilled water redistilled from a Pyrex still.

Table I gives complete data for one experiment. The temperature was 20°C., and a mixture of 5 per cent CO_2 in air was used. The manometer vessels were the rectangular type, illustrated in a paper by

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TABLE I

Respiration of Amoeba proteus

Vessel 6	Vessel 3
V_F = 7 cc., 14 mm.³ cells	v_f = 3 cc., 6 mm.³ cells
K_{O_2} = 0.56	k_{O_2} = 0.96
K_{CO_2} = 1.15	k_{CO_2} = 1.22
ΔH = 09.9 mm. in 130 minutes	Δh = −1.7 mm. in 130 minutes
X_{O_2} = −4.5 mm.³	
X_{CO_2} = +4.35 mm.³	
Q_{O_2} = −1.6	

the writer (1929), without alkali well. For a detailed explanation of the technique, see Warburg (1926). The notation of Table I is as follows:

V_F = volume of cell suspension.
K_{O_2} = constant of the vessel for oxygen.
K_{CO_2} = constant of the vessel for carbon dioxide.
Δh = pressure change on the manometer.
X_{O_2} = oxygen absorbed.
X_{CO_2} = carbon dioxide evolved.

Capital letters refer to the vessel with the large volume of cell suspension, small letters to the one with the small volume of cell suspension. The vessels contained suspensions of the same density, *i.e.*, the same volume of cells per cc. X_{O_2} and X_{CO_2} were calculated from these formulae:

$$X_{O_2} = \frac{\Delta H K_{CO_2} - \frac{V_F}{v_f} \Delta h k_{CO_2}}{\frac{K_{CO_2}}{K_{O_2}} - \frac{k_{CO_2}}{k_{O_2}}}$$

$$X_{CO_2} = \frac{\Delta H K_{O_2} - \frac{V_F}{v_f} \Delta h k_{O_2}}{\frac{K_{O_2}}{K_{CO_2}} - \frac{k_{O_2}}{k_{CO_2}}}.$$

X_{O_2} and X_{CO_2} refer to the gas exchange in Vessel number 6, which contained 14 mm.³ cells, over a period of 130 minutes, the time over

which the observation of the values of ΔH and Δh were read. For convenience in comparing with other figures, the value of X_{O_2} is reduced to the oxygen consumption in cubic millimeters per hour per 10 mm.^{3} cells, or Q_{O_2}, equal in this case to -1.6. This figure is much lower than figures published by the writer for green algae, where the value of Q_{O_2} ranged from about -5 to -10. Adolph (1929, p. 313) gives an average value for the oxygen consumption of freshly isolated frog skin, of 133 mm.^{3} O_2 per gm. of fresh weight. Assuming the density of the tissue to be close to 1, frog skin has, according to Adolph's figures, a Q_{O_2} of about -1.33, very close to the writer's figure of -1.6 for *Amoeba*.

Attempts were made to demonstrate anaerobic metabolism, by suspending the cells in media containing glucose or bicarbonate or both, and with an atmosphere of nitrogen which had been passed over red-hot copper to remove oxygen. No anaerobic metabolism was found.

Although it would undoubtedly be of interest to study the characteristics of *Amoeba* respiration, the writer did not undertake further experiments. Owing to the small gas exchange, much larger amounts of cells would be necessary than were available at one time.

II

Experiments with Blepharisma

Blepharisma was available in larger quantities, and consequently was better adapted for these experiments. The cells were free from other protozoans, and bacteria were removed by washing on the centrifuge. Measurements were made with cells suspended in distilled water from a Pyrex still, and in dilute salt solutions. Although the rate of respiration was about the same in either case, it remained constant in salt solution and fell off rapidly in distilled water. Only those experiments made with cells suspended in salt solution are recorded here.

The salt solution was prepared freshly from stock solutions for each experiment. When mixed, its composition was as follows:

Redistilled Water	1 liter
NaCl	500 mg.
KH_2PO_4	25 mg.
$CaCl_26H_2O$	100 mg.

In this solution *Blepharisma* cells will live and remain active for several hours without showing any signs of injury. Nothing was found which when added to this solution would cause any substantial increase in respiration. The addition of 1 per cent glucose caused a 10 to 20 per cent increase in oxygen consumption. Peptone in a concentration of 1 mg. per cc. caused a slightly greater increase.

TABLE II

Respiration of Blepharisma

Vessel 6, 87 mm.³ cells	Vessel 3, 37.5 mm.³ cells.
V_p = 7 cc.	v_f = 3 cc.
K_{O_2} = 0.56	k_{O_2} = 0.96
K_{CO_2} = 1.15	k_{CO_2} = 1.22
ΔH = −13 mm. in 25′	Δh = −1.7 mm. in 25 min.
X_{O_2} = −13.1	
X_{CO_2} = +11.7	
Q_{O_2} = −3.6	

Table II records the details of an experiment to determine the oxygen consumption and carbon dioxide production of a sample of *Blepharisma* cells. The notation and method of calculation is the same as for Table I. The temperature was 20°C.

The rate of gas exchange indicated in Table II remained constant for a period of three hours. As in the case of *Amoeba*, the respiratory quotient, $-\frac{X_{O_2}}{X_{CO_2}}$, is nearly equal to unity. The value given for Q_{O_2}, −3.6, is much greater than the figure for *Amoeba*. −3.6 was the lowest value obtained for *Blepharisma*, the average being around −5. With some samples of cells, Q_{O_2} was found to be as high as −7 or −8. These values compare favorably with figures of the writer, for green algae cited above. But they are much smaller than the figures given by Adolph (1929, p. 269) for *Colpoda*. He states that a unit volume of cells uses in an hour four times its volume of oxygen. This means a value of −40 for Q_{O_2}. Adolph says that his figures are more or less in agreement with the values found by other workers for protozoan respiration.

Blepharisma shows a measurable anaerobic metabolism. This is manifest only in the presence of bicarbonate, showing that an acid is evolved under anaerobic conditions, displacing CO_2 from the bicarbonate.

The demonstration of anaerobic metabolism was made as follows:

To 50 cc. salt solution were added 2 cc. of $\frac{M}{10}$ $NaHCO_3$. 80 mm.^{3} of fresh *Blepharisma* cells were washed in this solution and pipetted into Vessel 6. V_F was equal to 7 cc. The gas-space was swept out with nitrogen which had been passed over hot copper to remove oxygen. Nitrogen was passed through for about 5 minutes. This proved long enough to wash out the oxygen dissolved in the cell suspension without removing the CO_2 from the bicarbonate. Controls were made by adding the bicarbonate after saturation with nitrogen.

In the experiment referred to, 80 mm.^{3} cells in an atmosphere of nitrogen at 20°C. showed an increase of pressure of 11 mm. per hour. Assuming this to be due entirely to evolved CO_2, *i. e.*, assuming that nitrogen does not enter into the metabolism, this indicated an evolution of 12.5 mm.^{3} of CO_2 from the bicarbonate per hour. Upon returning the cells to an atmosphere of air, they showed normal respiration. Glucose did not affect the anaerobic metabolism.

Blepharisma cells are colored bright red, and it was thought they might show some special behavior toward light. However, no change in oxygen consumption could be detected when cell suspensions were illuminated by an incandescent lamp or by the light of a Pyrex mercury arc.

SUMMARY

1. The respiration of *Amoeba proteus* was measured. 10 c. mm. of cells were found to use about 1.6 mm.^{3} of oxygen per hour at 20°C. The respiratory quotient was found to be nearly unity.

2. No anaerobic metabolism was found for *Amoeba*.

3. The respiration of *Blepharisma* was found to be from 3 to 7 mm.^{3} oxygen per hour for 10 mm.^{3} cells. The respiratory quotient was about 1.

4. *Blepharisma* was shown to have a definite anaerobic metabolism. 80 mm.^{3} cells caused the evolution of 12.5 mm.^{3} carbon dioxide per hour at 20°C. in the presence of bicarbonate.

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SOME PROPERTIES OF THE PIGMENT OF BLEPHARISMA

By ROBERT EMERSON*

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(Accepted for publication, August 17, 1929)

In connection with measurements of the metabolism of *Blepharisma* described in the preceding paper, the writer made certain observations on the red pigment of this organism. The pigment of the intact organism defies extraction with any of the common organic solvents, but it may easily be obtained in solution by macerating fresh cells in clean quartz sand and extracting with 90 per cent ethyl alcohol.

The alcoholic extract behaves like an indicator. When neutral or acid, it is bright red, the color disappearing in alkali. The turning point is quite near neutrality, and only a slight degree of alkalinity is necessary to make the color-change complete. This may be brought about by adding a drop of M/10 sodium bicarbonate solution to 10 cc. of pigment solution. Perceptible changes in depth of color may be caused by merely varying CO_2 tension.

Fig. 1 shows the absorption curve of the extract of 108 mm.^{3} of cells made up to 10 cc. in ethyl alcohol. The curve was determined on a König-Martens spectrophotometer. ϵ, the extinction coefficient, is plotted against λ, the wave-length in $\mu\mu$. The spectrophotometer readings ϕ_1 and ϕ_2 are shown with their accompanying values of λ and ϵ in Table I.

The curve might be compared with the figure published by E. Ray Lankester in 1873 for the absorption spectrum of the unextracted pigment of *Stentor caeruleus*. Since stentorin is blue and the pigment of *Blepharisma* red, their absorption spectra would not be expected to coincide. But the three maxima shown by the *Blepharisma* pigment in the short-wave-length end of the spectrum might be said to correspond to the three absorption bands of stentorin in the long-wave-length end of the spectrum.

* National Research Council Fellow.

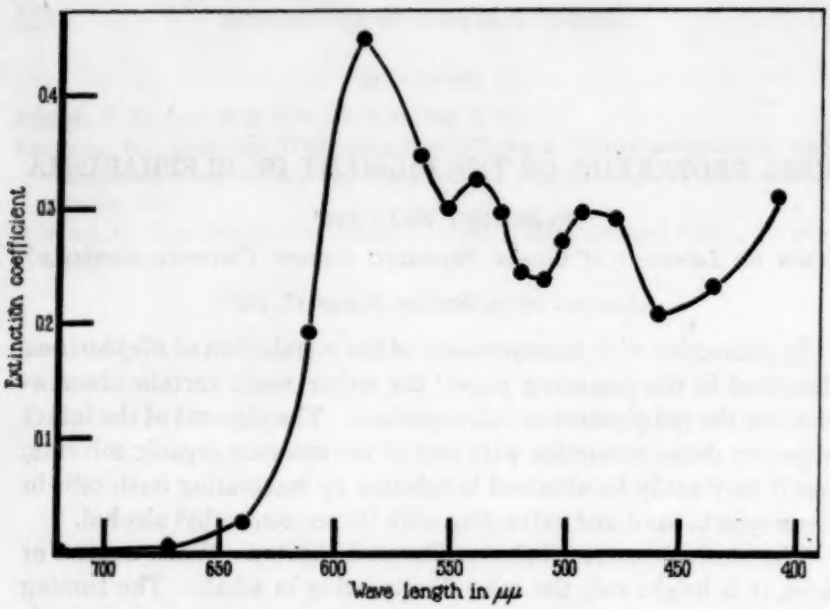


FIG. 1. Curve showing the absorption spectrum of the extracted pigment of *Blepharisma*.

TABLE I

Figures for the Absorption Spectrum of Blepharisma Pigment

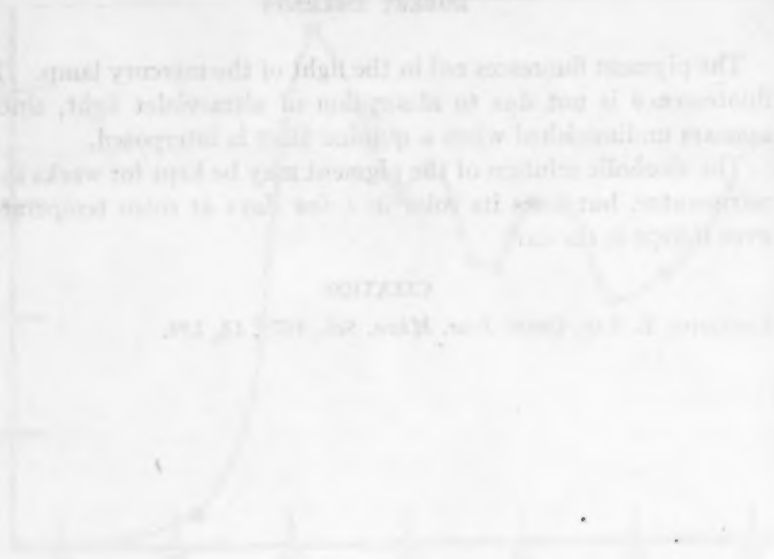
λ (μμ)	ϕ_1	ϕ_2	ϵ
720	42.0	42.0	0
662	41.2	41.2	0
630	42.5	41.0	0.023
602	48.0	36.0	0.186
579	55.7	28.2	0.457
564	53.1	30.6	0.353
552	51.0	31.8	0.300
540	52.0	31.2	0.325
529	51.0	32.0	0.296
520	49.2	33.4	0.245
510	48.9	33.5	0.238
502	49.9	32.5	0.271
494	51.1	32.0	0.297
479	50.7	32.0	0.291
460	Interpolated from another set of cells....		0.208
436			0.232
408			0.308

The pigment fluoresces red in the light of the mercury lamp. This fluorescence is not due to absorption of ultra-violet light, since it appears undiminished when a quinine filter is interposed.

The alcoholic solution of the pigment may be kept for weeks in the refrigerator, but loses its color in a few days at room temperature, even if kept in the dark.

CITATION

Lankester, E. Ray, *Quart. Jour. Micro. Sci.*, 1873, **13**, 139.



ON THE BEHAVIOR OF NICKEL CARBONATE IN RELATION TO PHOTOSYNTHESIS

By ROBERT EMERSON*

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(Accepted for publication, July 4, 1929)

Professor E. C. C. Baly (1927, 1928, 1929) claims to have synthesized carbohydrates by the action of visible light on an aqueous suspension of finely divided nickel carbonate saturated with carbon dioxide. He describes the production of weighable amounts of carbohydrate, of the order of 1 gm., by the action of 50 gm. of nickel carbonate.

To produce 1 gm. of glucose from carbon dioxide and water, approximately 0.95 gm. carbon dioxide is necessary. Under standard conditions this amount of carbon dioxide has a volume of 480 cc. One ten thousandth of this amount (48 c. mm.) would be easily measurable in a Barcroft-Warburg manometer, of the type commonly used.

It should therefore be possible to determine whether the appearance of Baly's carbohydrate is accompanied by the disappearance of a corresponding amount of carbon dioxide, and the production of a corresponding amount of oxygen. In any proof that artificial photosynthesis takes place, this should be an important step. It is omitted by Baly and his collaborators.

In this paper the writer describes experiments designed to demonstrate if possible the disappearance of carbon dioxide and the appearance of oxygen, when an aqueous suspension of nickel carbonate is exposed to visible light.

I

Preparation of the Nickel Carbonate

Nickel carbonate was prepared according to Baly's rather meager directions, by the electrolysis of a saturated solution of carbon dioxide in redistilled water with

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nickel electrodes.[1] Baly states that with three electrodes 8″ × 5″ × ⅛″ he used 220 volts, and "sufficient resistance intercalated to reduce the current to about 2 amps." He does not state the distance between his electrodes. The writer tried various distances, but was unable to make a similar system to conduct more than 0.5 amps. at 220 volts without any resistance. Using tap water, or commercial distilled water, the system conducted readily. It was concluded that some additional substance must have been present in Baly's system, to give such a high conductivity. If the current density is too low, no nickel carbonate is formed. Traces of various electrolytes were added to redistilled water to make it conduct enough current to produce a good yield of nickel carbonate. Hydrochloric acid was found to be most satisfactory. 0.5 cc. of "C.P." hydrochloric acid diluted 1 to 10 was added to 3 liters of electrolyte.

The precipitated carbonate was centrifuged off, dried to 100°C., heated to 135°–140°C. for 30 minutes, powdered in an agate mortar, passed through a 200-mesh phosphor-bronze sieve, spread in a thin layer in a crystallizing dish covered to protect from dust, and just prior to use illuminated for 12 to 18 hours by a 100 watt lamp at about 7 inches distance.

The substance prepared in this way is surely a basic carbonate of nickel. Heating to 135°–140° turns it a darker green, and it loses CO_2 which it can readily take on again. Heating to higher temperatures blackens it, and all the carbonate is converted to oxide. To obtain some idea of the composition of the preparation, samples which had been dried at 100° and not heated to 135° were weighed into a crucible, ashed, and the loss in weight determined. Assuming the residue to be nickel oxide, the carbonate preparation was found to contain 40 per cent nickel. To determine the amount of CO_2 present in the carbonate, weighed samples were treated with normal sulfuric acid, and the evolved CO_2 measured manometrically. The preparation was found to contain 8.75 per cent CO_2. Gmelin-Kraut (Handbuch der anorgischen Chemie, Heidelberg, 1909, **5,** Part 1, 108–109, 7th edition) states that basic nickel carbonate is of variable composition, and gives as example 3 NiO, CO_2, $6H_2O$. This gives nickel 46.8 per cent, and CO_2 11.7 per cent, figures not differing greatly from those of the writer.

II

Manometric Experiments

Samples of recently irradiated powder were weighed into the bulbs *B* of vessels of the type shown in Fig. 1. The main chamber *C* was given 3 cc. of redistilled

[1] The electrodes were furnished through the courtesy of the International Nickel Company.

water saturated either with 100 per cent CO_2 or with a mixture of 5 per cent CO_2 in air. The vessels were connected with their manometers, filled with the gas used to saturate their contents, and shaken in a thermostat at 20°C. until equilibrium was reached. The contents of the bulb was then washed into the main chamber, without opening the system.

The behavior was followed in continuous light or dark, or in intermittent light, by frequent readings of the manometers. All preparations absorbed carbon dioxide rapidly, absorption being most rapid with 100 per cent CO_2 in air. After mixing, the rate of CO_2 absorption rose rapidly to a maximum, and then fell off slowly, continuing at a

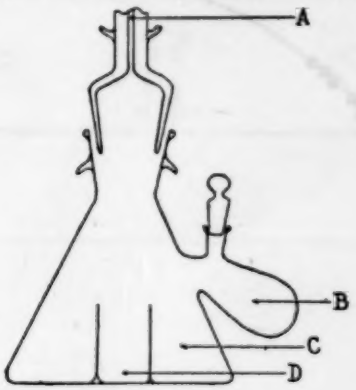

FIG. 1. Type of manometer vessel used. *A*, capillary connecting with manometer; *B*, bulb; *C*, main chamber of vessel; *D*, central well, in which sticks of yellow phosphorus were used.

decreasing rate for 4 or 5 hours. The course of absorption of a typical sample in 100 per cent CO_2 is shown in Fig. 2, total amount of CO_2 absorbed being plotted against time.

The curve shows the absorption of CO_2 by 8.5 mg. of nickel carbonate over a period of 5 hours and 10 minutes. At the end of this time, CO_2 was still being taken up perceptibly, though slowly. In all, 1825 c. mm. of CO_2 were absorbed by 8.5 mg. of carbonate in the period of observation.

The process is reversible at any point. Air may be substituted for 100 per cent CO_2, and the absorbed gas is then evolved. Much of the

CO_2 goes to forming a soluble nickel compound, possibly a bicarbonate, which can be precipitated if the CO_2 is pumped off.

A variety of combinations of CO_2 concentration, light intensity, and nickel carbonate samples were tried, but no effect of illumination could

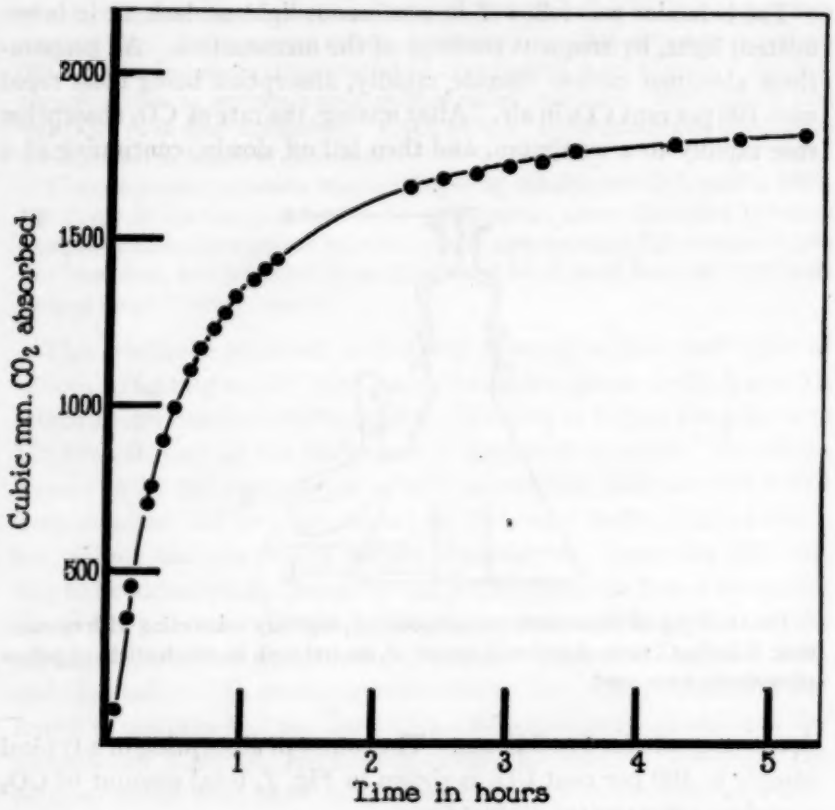

FIG. 2. Curve showing the absorption of CO_2 by 8.5 mg. nickel carbonate. Total CO_2 absorbed is plotted against time.

be observed. Illumination for short and long periods, of intensities from 10,000 to 100,000 Lux were tried, without any effect on the rate of CO_2 absorption.

Baly states that his preparations became inactive through the accumulation of oxygen. Some experiments were therefore tried with

small sticks of white phosphorous in the central well *D* of the manometer vessel, to absorb any possible oxygen, but no difference in behavior could be detected.

III

Gas Analysis Experiments

It was thought possible that a small photosynthesis might be concealed in the rapid absorption of CO_2 which was continuous in light or dark. Several analyses were made of the gas taken from above illuminated samples of nickel carbonate in water, in an attempt to demonstrate the production of oxygen during illumination.

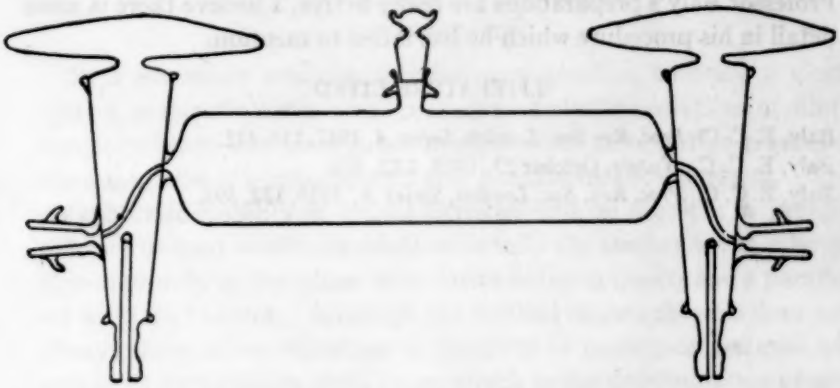

FIG. 3. Type of vessel used for gas-analysis experiments. The long stop-cocks are used to avoid having grease come in contact with the material under investigation.

Vessels of the type shown in Fig. 3, having a total volume of about 12 cc., were filled with 3 or 5 cc. of redistilled water saturated with the desired gas mixture. Just before closing the vessel, 50 to 500 mg. of nickel carbonate were added. The vessel was then shaken in either light or dark in a water thermostat for 2 to 5 hours. Analyses were made with an apparatus of the Haldane type on which one scale division was equal to 10 c. mm. No oxygen was ever detected as a result of illumination.

IV

Direct Test for Carbohydrates

The experiments described above were all carried out in closed vessels, whereas Baly's experiments were made in a continuous stream of carbon dioxide. A system similar to Baly's was devised as follows:

A glass cuvette of about 50 cc. capacity and 9 mm. distance between inside walls was filled with double-distilled water, and a stream of 100 per cent CO_2 was bubbled rapidly through. About 200 mg. of a fresh sample of nickel carbonate was added. The cuvette was illuminated by a 100-watt lamp 25 cm. away for five hours. The nickel carbonate was then centrifuged off, and the supernatant fluid concentrated *in vacuo*. The carbonate which precipitated out during concentration was separated off at intervals. The fluid was concentrated to a fraction of a cubic centimeter, and treated with sulfuric acid. There was no visible charring.

CONCLUSION

The writer was unable to prepare a sample of nickel carbonate which would give any indication of photosynthetic activity. If Professor Baly's preparations are really active, I believe there is some detail in his procedure which he has failed to mention.

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ELECTROKINETIC PHENOMENA

I. The Adsorption of Serum Proteins by Quartz and Paraffin Oil

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(Accepted for publication, July 24, 1929)

INTRODUCTION

It is extremely common in laboratory practice to bring a glass, quartz, or paraffin surface into contact with alkaline solutions of dilute serum proteins, but there is not much definite information regarding the adsorption of serum albumin and globulin by these surfaces. The cataphoretic mobility of quartz particles and oil droplets in protein sols will be used here to elucidate more fully the mechanism of adsorption of protein at the phase boundaries between quartz and a paraffin oil with electrolytes. Although the method of cataphoresis does not always show minor variations in quantity or quality of material adsorbed, it nevertheless gives an approach to the determination of any important gross selective adsorption. Quartz and oil droplets seem to adsorb gelatin and egg albumin non-specificially, but this is by no means true for all surfaces. Red cells of different mammals (1), for example, do not change their cataphoretic mobilities in the presence of small amounts of homologous or heterologous serum proteins. The presence of gelatin is equally ineffective. In fact, even in acid solutions of pH 3.6 uninjured red cells still retain their negative charge in the presence of gelatin (unpublished data). This means that the red cell surface is little if at all changed by the presence of relatively great excess of protein molecules.

The biological importance of comparing the cataphoretic mobility of quartz and paraffin oil in the presence of serum is best demonstrated by a few examples. The phagocytosis of inert particles like quartz, car-

bon, manganese dioxide, and so on is frequently studied without sufficient regard to the surface composition of the particles, neglecting the possibility that the particles may adsorb proteins in dilute solution to an extent that would alter the nature of the surface.* A parallel situation exists in studies of adhesiveness of leucocytes to various surfaces. Similarly it is claimed that the prolongation of the clotting time of blood by a paraffin surface is due to a difference between the adsorption of some blood constituent by glass and by paraffin (2).

Davis has studied the effect of serum proteins on the cataphoresis of glass particles (3). At the time of Davis' experiments, the method of micro-cataphoresis had not been so completely developed as it is today.† The differences between the data of this author and those to be reported here are probably due to the difference in method of measurement and in the nature of the adsorbing surfaces. Davis found that in isotonic buffers glass particles adsorb protein from about pH 2.0 to 8.0 (the range investigated), the charge being reversed at pH 4.5 in sols of sufficient concentration where the particles were presumably completely covered with protein.

Methods

Apparatus: The absolute mobility of the particles was determined in a modification of a type of apparatus devised by Northrop and Kunitz (4). An additional change was made in the construction of the non-polarizable electrodes. The agar plug previously used must be changed frequently—as often as once in five days or less. This introduces a serious loss of time. The ideal material would be porous and insoluble.‡ Thus far plaster of paris plugs, although not completely satisfactory, have been very useful. Sufficient powdered gypsum is put into the electrode vessels of the cataphoresis cell and mixed with water *in situ*. The porous plugs are then permitted to harden and dry for several hours. After filling the apparatus with saturated KCl solution, the solution in the apparatus is connected with the plaster plugs, the apparatus is inverted, and the electrode vessels are filled with saturated KCl. About 10 milliamperes are sent through the system for several hours with the electrode vessels still open. This apparently hastens the saturation of the pores of the plaster of paris plug with electrolyte. With a given voltage the current approaches a peak value, indicating that the KCl solution has permeated the entire system. The electrodes are then completed as described previously.

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*Jones, F. S., *J. Exp. Med.*, 1928, **47**, 245.

† Cemented cataphoresis cells were employed.

‡ Insoluble porous cements are being investigated.

The apparatus is not always free from streaming, which is particularly prone to occur with solutions of high conductance.* This streaming can usually be avoided by the following procedure. The apparatus is filled with some solution, preferably saturated KCl, and one stop-cock is turned, completing the connection between atmospheric pressure and the hydrostatic pressure within the electrode. After ten minutes equilibrium is usually reached and the same technic is employed on the other side. After each measurement the pressures are similarly readjusted for a few minutes. These procedures make possible the determination of small differences even though a system of high conductance is involved.

Suspensions: Crude quartz powder of small particle size (Eimer and Amend) was purified by heating with cleaning solution diluted with two parts of water, for about 30 minutes. The particles were allowed to settle and the excess of acid carefully decanted. A large amount of distilled water was added and the quartz was separated by a fine-pored Berkefeld filter. The powder was washed by about 500 cc. of distilled water and then boiled with an excess of fairly concentrated hydrochloric acid and allowed to settle. The filter was again employed to separate and wash the quartz. This washing was continued for several days. Very small particles were obtained by fractional sedimentation. It is important to sterilize the final suspension to prevent the growth of molds.

Emulsions of Nujol (a highly purified paraffin) were easily prepared by shaking equal volumes of Nujol and water or electrolyte very vigorously for about 15 minutes. The larger droplets separated out fairly quickly. Small amounts of this emulsion were added to the protein sols as needed, the volumes being identical in comparative experiments.

The number of particles of quartz and oil per $mm.^3$ in the final suspensions is difficult to determine with accuracy. The suspensions used in the final experiments were delicately cloudy and usually showed about 5–10 particles of 0.5μ to 5μ diameter per field (560 ×) with a blue filter. The same volume of a suspension of quartz particles from a standard suspension was always used.

Serum was obtained from clotted or defibrinated blood. Successive dilutions were made roughly by means of mixing cylinders.

Measurements were made at room temperature (20°–26°C.), in either 0.85 per cent or 0.35 per cent NaCl, the pH being fixed by dilute buffers and measured electrometrically.

EXPERIMENTAL

As is evident from Fig. 1, under the conditions of these experiments surfaces of particles of quartz and paraffin oil are extremely sensitive to

* It can be shown readily that this streaming (current = 0.0045 Amp., specific resistance of electrolyte = 70 Ohms; cross-section of cell = 0.07 $cm.^2$) is most probably produced by some mechanical deficiency in the apparatus rather than to heating by the current.

traces of protein in 0.8 per cent NaCl buffered by M/150 phosphate at pH 7.4.* With less than 1 part of human or rabbit serum in 100,000 parts of solution the mobility is markedly displaced, reaching a level which is fairly constant from 1:10,000 to 1:50. Similar results were obtained with glass particles. Under these conditions, then, the

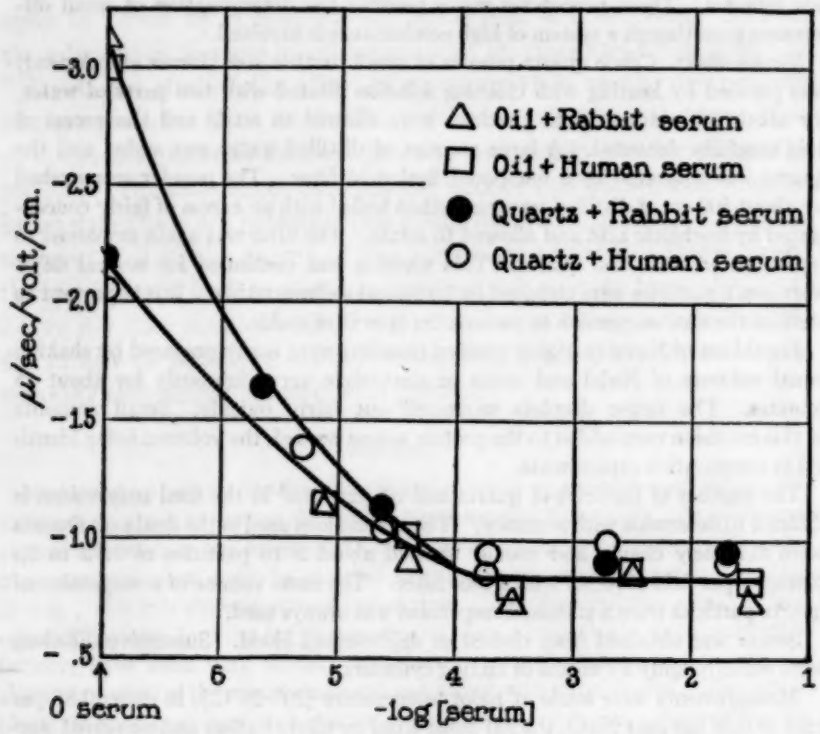


FIG. 1

hydrophilic surface of quartz and the hydrophobic surface of paraffin oil are very similarly attacked by some constituent of the serum, most likely a protein. These curves resemble those obtained with single proteins like gelatin. The data of Davis differ somewhat from those

* The value of about 1.0μ/sec./volt/cm. was obtained for many sera in the low dilutions. One serum gave a value closer to .5μ/sec./volt/cm. The reason for this anomalous behavior is unknown.

just presented. Davis found that in alkaline solutions protein was adsorbed by his glass particles with some difficulty. These differences may be due to differences in material employed or to experimental uncertainties. The experiments here recorded give excellent evidence that under certain circumstances probably a great part of surfaces of paraffin oil or glass are covered even at dilutions of serum of 1:100,000.

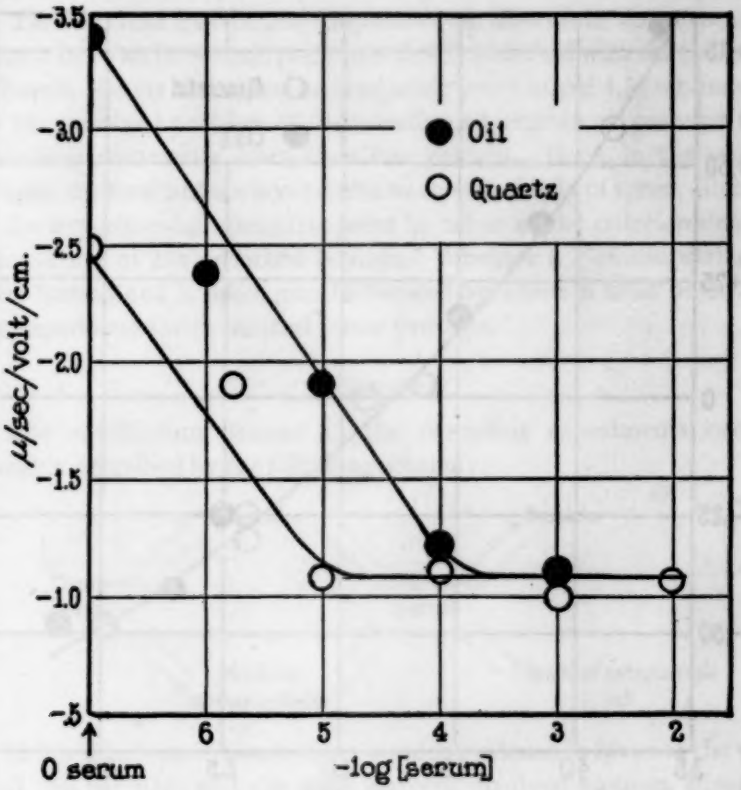


FIG. 2

Similarly, in the presence of leucocytes, red cells, and other systems giving rise to the merest trace of proteins, glass or paraffin oil surfaces must be considered as more or less subject to change, perhaps covered by a film of these proteins.

Fig. 2 shows the same phenomenon occurring at pH 9.3 (borate

buffer) in 0.85 per cent NaCl. Here, as at pH 7.4, the rather high alkalinity does not prevent adsorption of serum protein even in high dilution.

It was of interest to determine whether the parallelism in mobility of quartz and paraffin was observable over a wide range of pH including both sides of the isoelectric points of serum albumin and globulin.

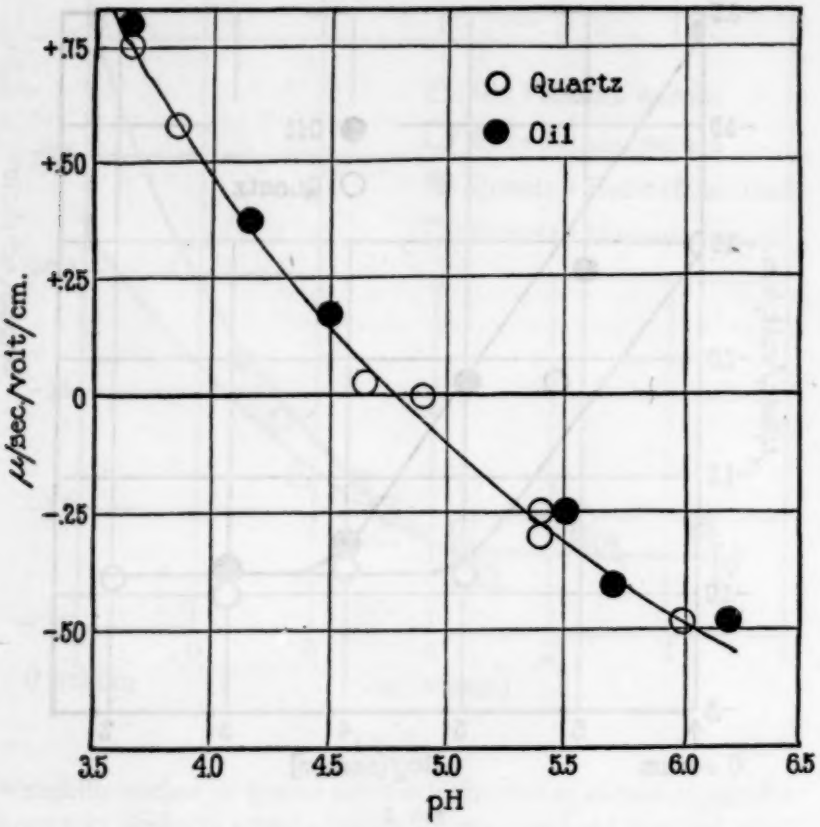


FIG. 3

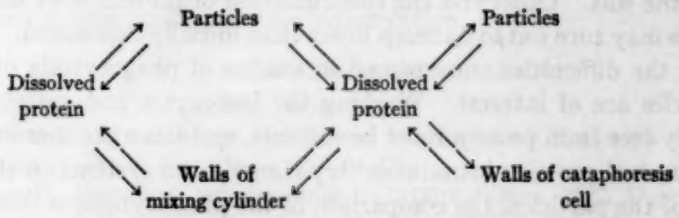
Suspensions of quartz and paraffin oil were studied in 1:50 serum diluted by 0.35 per cent NaCl buffered by N/100 acetate buffers, the ionic strength of the Na-acetate being held constant. In this system quartz and paraffin have fairly identical cataphoretic mobilities

(Fig. 3). Between pH 3.65 and pH 6.0 the curve of mobility as plotted against pH passes through an isoelectric point between 4.7 and 4.8, and resembles the titration curve obtained for serum albumin (5). It is obvious that the presence of electrolytes influences the course of such curves. The theoretical relationship correlating mobility of adsorbed protein and combining power for acids and bases has not yet been described.*

The fact that quartz and droplets of paraffin oil in the presence of serum have an isoelectric point practically identical with that of serum albumin (Davis had noted an isoelectric point of pH 4.5) reminds one of the unsolved problem of the selective adsorption of proteins from solutions containing more than one protein. Here, in the case of serum, the final surface layer seems to consist chiefly of serum albumin, if the experimental isoelectric point be taken as the criterion defining the nature of the adsorbed protein. Whether a globulin surface is first formed and is subsequently covered by albumin must be decided by experiments with purified serum proteins.

DISCUSSION

The equilibrium present in the preceding experiments can be roughly described by the following schema:



In low dilutions of serum there is much evidence in favor of the view that the particles and the glass surfaces involved have an adsorbed protein film which forms the interface at the water boundary. In dilute solutions certain complications are present. For example, in 1:1,000,000 dilution six or more different mixing cylinders are used. In each dilution succeeding the one in which a complete protein film is formed, unknown and varying amounts of protein are adsorbed by

* See Abramson, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1929, 26, 689.

the walls of the mixing cylinders. Thus the *quantity* of dissolved protein present in the final dilution is uncertain. Furthermore, the *nature* of the protein may change. Svedberg and Sjögren (6) have reported that when serum albumin is diluted, decomposition of the albumin molecules occurs. It is evident from the experiments reported here and elsewhere that the mobility (7) imparted by adsorbed protein micells to inert particles at a 1:10,000 dilution is very near that at a 1:100 dilution. What occurs at extremely high dilution is unknown. Another interesting phenomenon observed in this connection in protein sols at high dilutions, *e.g.* at $1:10^7$, with quartz particles in suspension, is the variation in speed amongst the various quartz particles. Some of the particles move as if no protein were present. Others have their velocities lowered slightly—but without the uniformity observable in more concentrated sols. This indicates an uneven distribution of the protein on the particles. When comparatively few protein molecules are available, the equilibrium between particle, vessel wall and dissolved protein is, therefore, most difficult to analyse.*

The practical applications of the preceding remarks in relation to the diluting of sera is apparent. It is advisable at high dilution, 1:1000 or over, to have as little glass surface as possible in contact with the sols. Otherwise the concentration of protein or of immune bodies may turn out to be much lower than initially calculated. Similarly, the difficulties encountered in studies of phagocytosis of inert particles are of interest. Washing the leucocytes and vessels completely free from protein must be difficult, and since the merest trace of adsorbed proteins introduces very complicated systems on the surface of the particles, the comparison of the phagocytosis of dissimilar surfaces such as quartz and paraffin oil in the apparent absence of serum is hardly feasible. If serum is present in sufficient excess so that it can be shown that the particles under investigation are completely and similarly covered by protein films (so that all the particles have the same cataphoretic mobility), the comparison may be more definite (8).

* In low (1:50) serum dilutions anomalous behavior has been rarely observed. For example, a quartz particle during the course of a day's experiments may be observed to be unaffected by the protein although all others in the systems studied were completely covered.

Since cataphoretic velocity is but one function of the particle ion atmosphere, differences in phagocytosis may conceivably still be present even though the cataphoretic mobilities of two different types of particles are the same.

SUMMARY

1. The effect of human and rabbit sera on the cataphoretic mobility of glass and quartz particles, and of paraffin oil droplets, was studied in serum dilutions (with 0.85 per cent NaCl) from 1:50 to 1:1,000,000, over a pH range of 3.6 to 9.3.

2. Under the conditions described, these various types of particles adsorbed protein partially or completely from the most dilute solution giving these particles electrokinetic properties characteristic of certain proteins, probably here those of serum albumin.

3. Quartz particles and paraffin oil droplets both have an isoelectric point between 4.7 and 4.8 in a 1:50 serum dilution.

4. The biological importance of these findings is discussed.

5. A non-polarizable electrode composed of Cu | $CuSO_4$ | $\left.\begin{matrix} CaSO_4 \cdot 2H_2O \\ Sat \cdot KCl \end{matrix}\right|$ is described for use with cataphoresis cells.

I am indebted to Professor L. Michaelis for much valuable advice received in connection with this investigation.

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THE SIZE OF BACTERIA AS THE CAUSE OF THE LOGARITHMIC ORDER OF DEATH

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The observation first[1] made by Madsen and Nyman (1907) and independently by Chick (1908) that bacteria killed by almost any cause die in an orderly way which has the same mathematical formulation as the monomolecular reactions, has been verified in a general way by most subsequent investigations. There have been differences of opinion regarding technique; it has been found that the reaction is not always strictly logarithmic, because the "reaction velocity" or the rate of disinfection is not constant but frequently decreases, and this could be accounted for by assuming a varied resistance of the different cells; there has been, in some experiments, the necessity of omitting the first few counts in order to get a reasonable agreement of the death rate. But all experiments made to prove or disprove the claim show that in a general way, the orderly death of bacteria is logarithmic, and that we are justified in speaking of a "logarithmic order of death."

Much less agreement could be obtained on the interpretation of this orderly process. The one extreme of explanation is the assumption that bacteria are small enough to act as molecules, and enter into reaction as any other large molecule would, and therefore must follow the mass law; the mass law reactions are logarithmic. Others scorn

[1] Falk (1923) states that Ikeda (1897) was the first to observe this agreement with the monomolecular law, but it seems to the author that he gives Ikeda too much credit. Ikeda found only that the ratio of times to bring about the same disinfection effect in different concentrations of the same disinfectant is the same. He does not mention any similarity with unimolecular reactions (see also Reichenbach, 1911 and 1922–23).

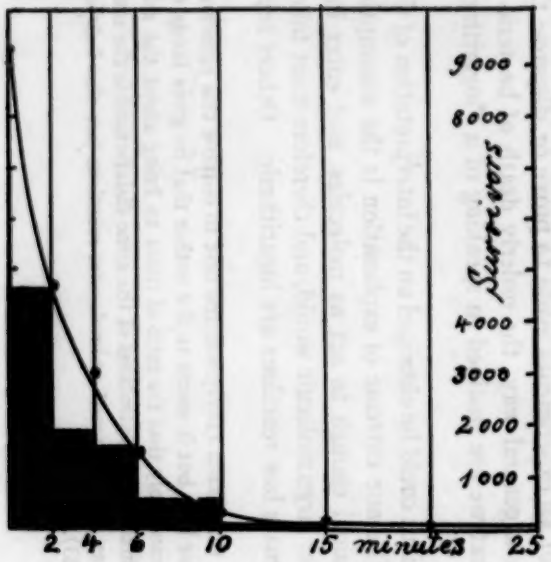

FIG. 1

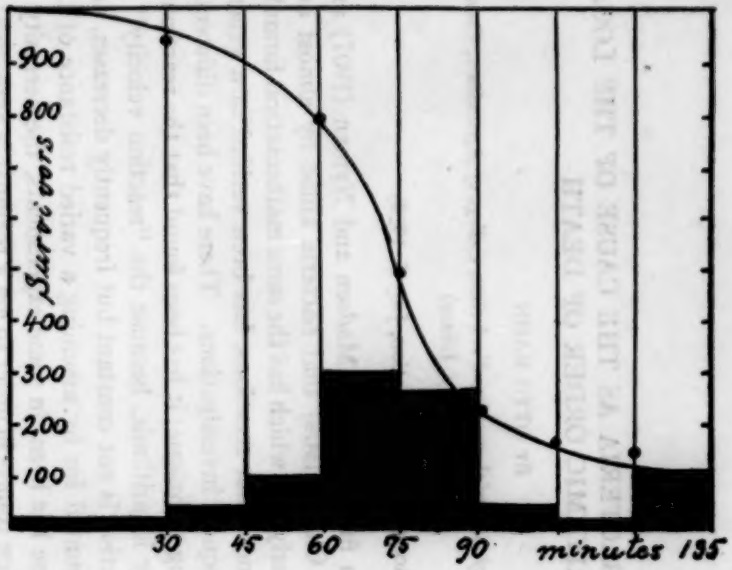

FIG. 2

FIG. 1. Death of spores of *B. anthracis* by $HgCl_2$. The black line demonstrates the numbers of survivors; the blocks represent the numbers of spores dying per unit time (2 minutes).

FIG. 2. Death of mustard seeds by $HgCl_2$. The black line demonstrates the numbers of survivors; the blocks represent the numbers of seeds dying per unit time (15 minutes).

the idea of comparing such large organisms (with flagella, cell wall, vacuoles and granules indicating a high complexity of composition) with molecules; they believe that the orderly process of death is simply a consequence of an orderly variation of resistance. It is not very easy, however, to fit the laws of chance to the actual facts of the order of death of bacteria, as shall be demonstrated by one of the first experiments on this order, by Madsen and Nyman (1907), and the first experiment for the explanation by variability, by Hewlett (1909).

Fig. 1 shows that the curve of survivors in the case of bacteria spores is distinctly logarithmic, while the survivor curve of the mustard seeds, killed by the same poison and washed with water and treated

TABLE I

Death Caused by Mercuric Chloride

A Spores of *B. anthracis* in 0.5 per cent $HgCl_2$				B 1,000 Mustard Seeds in 0.2 per cent $HgCl_2$			
Time	Survivors	Spores killed in 2 minutes	$\frac{1}{t}\log\frac{a}{b}$	Time	Survivors	Seeds killed in 15 minutes	$\frac{1}{t}\log\frac{a}{b}$
min.				*min.*			
0	9,500	—	—	30	940.0	(30)	0.0018
2	4,860	4,640	0.146	45	895.8	44.2	0.0016
4	2,964	1,896	0.126	60	790.6	105.2	0.0023
6	1,408	1,556	0.138	75	486.6	304.0	0.0052
10	304	552	0.149	90	220.6	266.0	0.0087
15	2.6	120	0.204	105	163.8	56.8	0.0087
20	1.8	0.3	0.186	120	146.0	17.8	0.0080
25	2.0	0	0.147	135	39.0	107.0	0.0117

with hydrogen sulfide exactly like the bacteria spores, is a typical inverted S-shape. More striking yet is the difference in the "death rate curves" shown by the black blocks and presenting the number of spores or seeds dying in each unit of time. The third difference is the "reaction velocity" or "rate of death" as shown by the K values in Table IA and IB, computed according to the general formula

$$k = \frac{1}{t}\frac{\log a - \log b}{0.434}$$

In Table IA, k is fluctuating but remaining constant within a very large error; in Table IB, however, the death rate is increasing continuously.

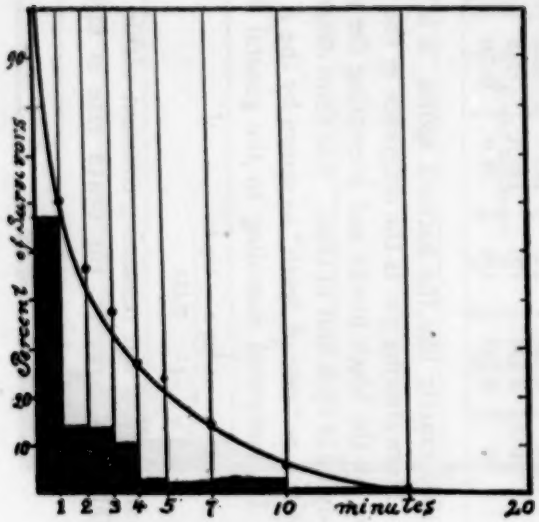

FIG. 3. Death of *Bact. typhosum* by exposure to 49°C.

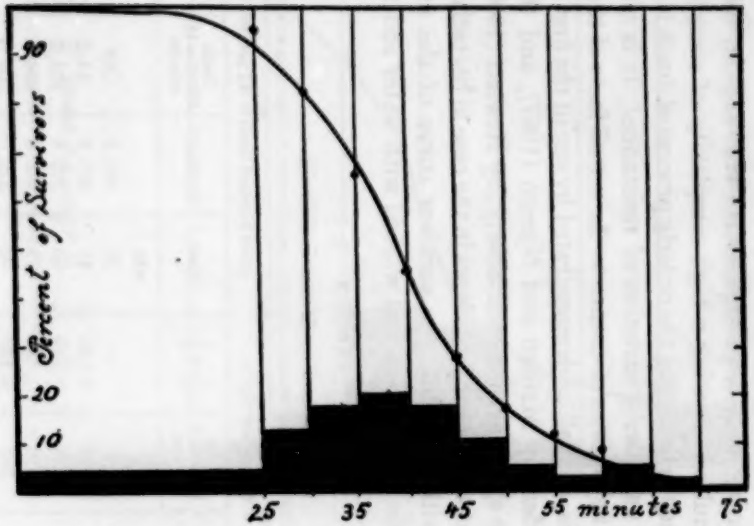

FIG. 4. Death of *Drosophila* by exposure to 39.45°C.

The black line demonstrates the numbers of survivors; the black blocks represent the percentage of organisms dying per unit time (1 and 5 minutes respectively).

Exactly the same differences between bacteria and multicellular organisms are found if they are exposed to heat, as shown in Table IIA (data by Chick) and B (data by Loeb and Northrop).

If the order of death as actually observed with bacteria is to be explained by variation in resistance, then this variation must be of a special kind. Variation of biological characters generally follows the laws of chance, and the black blocks of Figs. 2 and 4 show the general trend of such curves. Figs. 1 and 3 show how the distribution of resistance would have to be if an approximation to the logarithmic

TABLE II

Death Caused by Heat

A *Bact. typhosum* at 49°C.			*B* Fruit flies, at 39.45°C.		
Time	Survivors	Per cent dying per minute	Time	Per cent of survivors	Per cent dying each 5 minutes
min.			*min.*		
0.28	2,008	—	0	100	—
1.00	1,198	56.0	25	95.5	1.8
2.05	925	13.6	30	83.0	12.5
3	755	18.5	35	65.4	17.6
4	542	10.6	40	45.4	20.0
5	488	2.7	45	28.0	17.4
7	289	4.9	50	17.1	10.9
10	112.8	2.9	55	11.8	5.3
15	24.2	0.9	60	8.8	3.0
20	3.0	0.1	65	2.5	5.3
—	—	—	70	1.1	1.4
—	—	—	75	0.3	0.8

sequence were to be expected. The most sensitive organisms must exist in the largest numbers. That is difficult to explain. Reichenbach (1911) constructed a special theory of growth in order to fit these facts. He assumes that after each doubling of the cells, a certain percentage do not multiply further, but become dormant; these dormant cells are the more resistant the longer they remain in that state. Since even this did not give the correct logarithmic death process, a correction factor was introduced in addition. This assumption seems rather artificial, and does not explain why bacteria spores follow the logarithmic law of death just as accurately or even more so than the vegetative forms.

Neither of these two interpretations of the order of death, which Lee and Gilbert (1918) termed the "mechanistic" and the "vitalistic" theories, has been proved or disproved. No new viewpoint has been added during the last ten years, though the material pro and con has been increased. On account of this deadlock for such a long time, the interest in this question which seems, after all, of considerable biological significance has decreased.

To illustrate how the "mechanists" and "vitalists" each adhere strongly to their theories, a few quotations are given:

Loeb and Northrop (1917): "When we plot the number of flies which die during successive days in terms of percentage of the original number of flies we get that curve of the death rate usually given in life insurance statistics, namely, a probability curve, the ascending branch of which is a little steeper than the descending branch . . . . Miss Chick has stated that bacteria are killed by disinfectants at a rate corresponding to that of a monomolecular chemical reaction, *i.e.* that in each interval of time the same percentage of individuals alive at this time is killed. She was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep. The agencies used by her for killing the bacteria were so powerful that the ascending branch became almost a vertical line, thus escaping attention. Hence she noticed usually only the less steep descending branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time."

Fulmer and Buchanan (1923) (Summary): "It is believed that such resemblances as have been found between such curves (survivor curves plotted against time) and monomolecular reactions or logarithmic curves are superficial and fortuitous. Any method therefore of evaluating disinfecting power based upon such a concept must prove misleading.

"Variations in resistance of individual cells and the distribution of such variations must be regarded as of fundamental importance in accounting for rates of death of organisms."

Lee and Gilbert (1918) (Summary): "It has been proposed to group all these theories (of disinfection) into two classes, namely "vitalistic theories" and "mechanistic theories" . . . . . In view of the experimental evidence which has been presented, disinfection, in the opinion of the authors, must be regarded as an orderly time process which is closely analogous to chemical reaction,—the microorganisms and the disinfectant being regarded as the respective reagents. A definite logarithmic relationship between velocity of disinfection and concentration has been found to exist in all cases investigated. . . . The "theory of graded resistance" as advanced by Eijkman, Hewlett and Reichel has been reviewed, and attention has been directed to the fact that biological characteristics are distrib-

uted as a rule in a manner quite different from that which they have assumed in formulating their theory. In view of these observations, the authors are led to the conclusion that the logarithmic nature of the disinfection process is due to a general *similarity* of the individuals in a given pure culture of microorganisms rather than to a *dissimilarity* of the individuals as postulated in the theories of graded resistances by the supporters of the vitalistic theory."

Cohen (1922): "Subjecting organisms of the colon-typhoid group to mild lethal conditions under moderate temperatures and hydrogen ion concentrations tends to magnify the induction period prior to mortality at the maximum or logarithmic rate. . . . The period of induction is decreased by higher acidity and by higher temperature. It appears to have a duration inversely proportional to some exponent of the temperature. It is analogous to the induction period occurring in chemical reactions. . . . The mortality of bacteria whether by strong disinfectants or by milder agents follows the laws of logarithmic decline. It is shown that the course of the disinfection process can be expressed by mathematical relations comparable to those used in dealing with monomolecular chemical reactions."

A compromise theory similar to that of Lee and Gilbert was attempted by Falk (1923): "Indeed, it is our opinion that the findings of Chick, Brooks, Loeb and Northrop, and of Cohen have all shown that when the material which is being studied is—with respect to the reaction—physiologically homogeneous the course of disinfection, hemolysis, mortality etc. simulates the well-known logarithmic curve of mass action chemical processes. When the viable material is lacking in homogeneity, *i.e.* as between young and old cells, or as between vegetative and spore forms in Dr. Chick's work, the curve which describes the course of the reaction varies from the logarithmic."

This theory seems to be given up later by Falk in favor of an explanation by multimolecular reaction as it appears from the following quotation:

Falk and Winslow (1926): "Where disinfection does not follow a logarithmic course, and is not to be described by the unimolecular equation, the course of the process may sometimes be described by the equation of a bi-, tri-, or higher multimolecular reaction. Such an explanation may render unnecessary the assumption of variability in biological resistance to account for deviation from a logarithmic mortality curve."

Whenever two groups of thorough research workers hold such opposed views as in this case, it is fairly safe to assume that some essentially new principle is involved which none of the two parties realized.

A comprise seems difficult, but it is so largely because the "mechanists" do not pay sufficient attention to the deviations from the strict logarithmic law, while the "vitalists" refuse largely to acknowledge the facts, and base their refusal upon an analogy with multicellular organisms which may not be altogether correct, as shall be proved

presently. One more reason for considerable confusion in disinfection experiments is the difficulty in obtaining identical death rates with subcultures of the same strain on different days. The death rates vary so enormously that an unknown factor, perhaps of catalytic nature, must be assumed. But this has really nothing to do with the order of death; it affects the rate only, and the present discussion is altogether limited to the order of death.

The attempt to explain the logarithmic order of death as a consequence of the small size of bacteria shall be limited here to the death by heat. It is based upon the assumption that death is caused by some chemical change taking place in the cell, and that this chemical process as such follows the same laws as all other chemical processes. Possibly, this reaction is a coagulation of a certain cell protein, or it is the inactivation of an enzyme essential for life, or of some other thermolabile cell compound. The heat coagulation of proteins follows the mass law, and is a monomolecular process (Chick and Martin, 1910); so is the heat destruction of enzymes (Tammann, 1895). It would be not at all surprising that the chemical process causing death by heat is monomolecular. This process, whatever it be, is called by Brooks (1918) the "fundamental reaction."

Thus far, probably, the vitalists will agree. But they will say at once that as long as all cells are alike, the same reaction must proceed in exactly the same way in all cells, and if 20 per cent of the protoplasm in one cell is coagulated, there must be 20 per cent coagulated in everyone of the cells. They must all die at exactly the same moment if they are alike, and the fact that they do not all die simultaneously is the best proof for variation in resistance. This argument can be met by introducing size as a factor.

Let us consider this process of heat coagulation in more detail. If we place a beaker with an albumin solution in a water bath of 65°C., the mass of protein will be gradually coagulated according to the mass law. If this solution were divided by a partition into two independent halves, it will be generally agreed upon that, upon heating in the water bath, the processes will be exactly parallel in the two liquids. If we divide the mass of protoplasm evenly into 1000 separate units, the process will be parallel in each of the units, and when half of the protein is coagulated in one of them, we are certain that exactly the same has happened in each of the other 999.

And yet, this parallelism is not unlimited, because finally, if we continue to divide the total mass of protein, there will be only one molecule left to each division. What will happen when this liquid divided up into these smallest units, each containing only one molecule of protein, is placed into the water bath of 65°C.? The molecules will react exactly in the same way as if they were in a continuous liquid, as if there were no partitions; they will follow the mass law. The division into smallest units can make no alteration in the process, because the protein molecules do not react with each other, at least not primarily. They either react with water in which they are suspended, or they give off water; neither of these processes could be altered by partitions in the medium.

This means, then, that the sum of all these units would have to follow the logarithmic law of mass action. In this case where each unit contains only one reacting molecule, some molecules will react faster then others, and consequently, some units will contain changed molecules and some will not, even though they were exactly alike at the start, and had been treated in exactly the same way. This division into the smallest possible units makes the mass law a fallacy. The mass law holds true only where the number of reacting molecules is practically innumerable. If the number of molecules is limited, the laws of chance enter. It will be shown later than even with 100 reacting molecules per cell, the chance is not altogether excluded.

The extreme theory that the entire protoplasm is one giant molecule need not be assumed for our purpose here. Doubtless the smallest bacteria cell contains many protein molecules though they may be interlinked somehow. These molecules are not all alike. There must be some specialization corresponding to that of the chromosomes in plant cells. Each section of each chromosome is different as the geneticists have shown. It is not a very bold theory to assume that one of these sections is essential for growth or multiplication, and with its destruction, the cell loses the power of multiplication and is considered dead according to the plate count technique of the bacteriologist.

This same order would hold true if there were several such essential molecules in each cell, and the destruction of any one of them would cause death. If, however, two molecules of this most essential type

must be destroyed before multiplication ceases, then another law enters. On the following pages, an equation for the order of death is developed for any number of reacting molecules.

If a bacteria per unit space (*e.g.* per cc.) are exposed to some definite unfavorable temperature which will inactivate m molecules of protoplasm in m cells per unit time, the rate of inactivation of the molecules is $\frac{m}{a}$. The portion not acted upon is $1 - \frac{m}{a}$.

If the destruction of *one* molecule per cell would cause the bacterium to die the numbers of survivors and of dead bacteria would be

After the time	Survivors	Dead cells
0 units	a	0
1	$a\left(1 - \frac{m}{a}\right)$	m
2	$a\left(1 - \frac{m}{a}\right)^2$	$m\left(1 + 1 - \frac{m}{a}\right)$
3	$a\left(1 - \frac{m}{a}\right)^3$	$m\left[1 + \left(1 - \frac{m}{a}\right) + \left(1 - \frac{m}{a}\right)^2\right]$

We substitute for the expression $\left(1 - \frac{m}{a}\right)$ which returns again and again, the letter q, and obtain for the time n the expressions

n	$a\,q^n$	$m(1 + q + q^2 + q^3 + \ldots + q^{n-1})$
		$= m\left(\frac{1 - q^n}{1 - q}\right) = a(1 - q^n)$

The decrease of the survivors is logarithmic; $q = 1 - \frac{m}{a}$ is the proportion of cells not acted upon in unit time.

The next case would be the assumption that the cell can recover if only one molecule is destroyed, but will die if two molecules are inactivated. We proceed as above:

Time	Uninjured	Injured	Dead
0	a	0	0
1	aq	m	0

Time	Uninjured	Injured	Dead
2	aq^2	$2mq$	$\frac{m^2}{a}$
3	aq^3	$3mq^2$	$\frac{m^2}{a}(1+2q)$
n	aq^n	nmq^{n-1}	$\frac{m^2}{a}\left[1+2q+3q^2+..+(n-1)\,q^{n-2}\right]$ $= a[1+nq^{n-1}+(n-1)\,q^n]$

If we assume that 3 molecules have to be destroyed before the cell cannot recover, we will have four groups of cells:

Time	Uninjured	½ Injured	¼ Injured	Dead
o	a	0	0	0
1	aq	m	0	0
2	aq^2	$2mq$	$\frac{m^2}{a}$	0
3	aq^3	$3mq^2$	$\frac{3m^2}{a}q$	$\frac{m^2}{a^2}$
4	aq^4	$4mq^3$	$\frac{6m^2}{a}q^2$	$\frac{m^2}{a^2}(1+3q)$
n	aq^n	nmq^{n-1}	$\frac{n(n-1)}{2}\frac{m^2}{a}q^{n-2}$	$\frac{m^2}{a^2}\left[1+3q+6q^2+\ldots\right.$ $\left..+\frac{(n-1)(n-2)}{2}q^{n-2}\right]$

It is desirable to get a general formula for any number of molecules r necessary to be destroyed to insure the death of the cell. Thanks to the experience and kind assistance of Dr. W. A. Hurwitz, of the Department of Mathematics, Cornell University, such a general formula could be derived by studying the development of the formulae so far mentioned. If we chose, *e.g.* the number of dead cells after the

time n which we may call Dn, we find the following regularity in the development:

1 molecule: $m[1 + q + q^2 + q^3 + \ldots\ldots + q^{n-2} + q^{n-1}] = Dn^{(1)}$

2 molecules: $\frac{m^2}{a}\left[1 + 2q + 3q^2 + \ldots\ldots\ldots (n-1)\, q^{n-2}\right] = Dn^{(2)}$

3 molecules: $\frac{m^3}{a^2}\left[1 + 3q + 6q^2 + \ldots\ldots \frac{(n-1)\,(n-2)}{2}\, q^{n-3}\right] = Dn^{(3)}$

The regularity of development consists in this: the expression in the second equation after $\frac{m^2}{a}$, is the first derivative of the corresponding expression in the preceding line, *i.e.* the factor of m; in the same way, with 3 molecules, the sum $[1 + 3q + \ldots]$ is one-half of the derivative of the preceding expression, $[1 + 2q + \ldots]$. If we continue this systematic development, and call the total sum of the expression for 1 reacting molecule $= f(q)$, we find:

$$Dn^{(1)} = mf(q)$$

$$Dn^{(2)} = \frac{m^2}{a} f'(q)$$

$$Dn^{(3)} = \frac{m^3}{2\,a^2} f''(q)$$

$$Dn^{(4)} = \frac{m^4}{a^3\,(3)!} f'''(q)$$

$$Dn^{(r)} = \frac{m^r}{a^{r-1}} \frac{1}{(r-1)!} f^{(r-1)}(q)$$

The original $f(q)$ was found to be $1 + q + q^2 + q^3 + \ldots + q^{n-1}$. Summarized, this gives

$$f(q) = \frac{1 - q^n}{1 - q}$$

Thus, for the case that inactivation of only one molecule is sufficient to cause death, we have the equation

$$(1 - q)\, f(q) = 1 - q^n$$

This equation, differentiated, gives

$$(1 - q) f'(q) - f(q) = - nq^{n-1}$$

If this is differentiated again, we obtain

$$(1 - q) f''(q) - f'(q) - f'(q) = - n(n - 1) q^{n-2}$$

$$(1 - q) f''(q) - 2f'(q) = - n(n - 1) q^{n-2}$$

The next differentiation will give us the expression for the case where 4 molecules must be destroyed to cause death:

$$(1 - q) f'''(q) - 3f''(q) = - n(n - 1) (n - 2) q^{n-3}$$

The order of progression is plain, and we can write the reaction equation for r molecules:

$$(1 - q) f^{(r-1)}(q) - (r - 1)! f^{(r-2)}(q) = - n(n - 1) (n - 2) \ldots (n - r + 2) q^{n-r+1}$$

This equation is multiplied with $\dfrac{m^r}{a^{r-1} (r - 1)!}$

$$\frac{m(1 - q) f^{(r-1)}(q)}{a^{r-1} (r - 1)!} - \frac{f^{(r-2)}(q) m^r}{(r - 2)! a^{r-1}} = \frac{- n(n - 1) (n - 2) \ldots (n - r + 2)}{(r - 1)!} q^{n-r+1} \frac{m^r}{a^{r-1}}$$

We now substitute in this equation the value for $Dn^{(r)}$ from the preceding page, and also the value for $Dn^{(r-1)}$:

$$Dn^{(r)} (1 - q) - \frac{m}{a} Dn^{(r-1)} = \frac{- n(n - 1) (n - 2) \ldots . (n - r + 2)}{(r - 1)!} \cdot \frac{m^r}{a^{r-1}} \cdot q^{n-r+1}$$

Since $1 - q = \dfrac{m}{a}$, we get

$$Dn^{(r)} = Dn^{(r-1)} - \frac{n(n - 1) (n - 2) \ldots . (n - r + 2)}{(r - 1)!} \cdot \frac{m^{r-1}}{a^{r-1}} \cdot q^{n-r+1}$$

Thus, for any r-value, the number of dead cells after the time n is given by the number of the dead cells for the reaction of the next lower order; if we just continue this, we must finally come to $r = 1$

and this case is known. The number of dead cells can therefore be computed for any value of r and n. Substituting the letter C_r for the long expression to be substracted from $Dn^{(r-1)}$, we obtain

$$Dn^{(r)} = Dn^{(r-1)} - C_r$$

$$Dn^{(r-1)} = Dn^{(r-2)} - C_{r-1}$$

$$Dn^{(r-2)} = Dn^{(r-3)} - C_{r-2}$$

$$\vdots$$

$$Dn^{(2)} = Dn^{(1)} - nmq^{n-1}$$

The number of dead cells is then really the total sum of all the C_r-values substracted from the last member $Dn^{(1)}$, which is known to equal $a\ (1 - q^n)$ (p. 188). This gives the following value for $Dn^{(r)}$:

$$Dn^{(r)} = a(1 - q^n) - nmq^{n-1} - \frac{n(n-1)}{2}\frac{m^2}{a}q^{n-2} - \ldots$$
$$- \frac{n(n-1)\ldots(n-r+2)}{(r-1)!} \cdot \frac{m^{r-1}}{a^{r-2}} \cdot q^{n-r+1}$$

We substitute

$$1 - q = p = \frac{m}{a}$$

$$Dn^{(r)} = a\left(1 - q^n - nq^{n-1}p - \frac{n(n-1)}{2}q^{n-2}p^2 - \ldots\ldots\right.$$
$$\left. - \frac{n(n-1)\ldots\ldots(n-r+2)}{(r-1)!} \cdot q^{n-r+1} \cdot p^{r-1}\right)$$

This is the final expression for the total number of dead cells after the time n, when r molecules per cell must be destroyed before the cell is dead.

This final equation does not mean very much to the average biologist (including the author) who is not trained along statistical lines, and the only way to make it intelligible is to apply it to some simple case.

Unfortunately, it has not as yet been possible to condense this long expression to a simpler form, and the application means therefore a very tedious computation of many data. By choosing very simple conditions, however, the amount of work can be reduced a little.

The following example is calculated on the assumption that 1,000,000 cells are acted upon by some harmful influence at such a rate that 90 per cent of the protoplasm molecules are inactivated per unit time. In the terms of our equation, this means

$$a = 1{,}000{,}000$$

$$p = \frac{m}{a} = 0.9$$

$$q = 1 - \frac{m}{a} = 0.1$$

n is the unit of time. In Table III, the number of survivors is calculated for the end of each unit of time until there is less than one living cell left. This calculation is carried through for successive r-values from 1 to 12; that means, for the assumption that the destruction of 1, 2, 3, etc. molecules means the death of the cell. The table gives the total number of survivors, and the cells dying per unit time.

The cells dying per unit time are also plotted in the block curves (Fig. 5) which will show to most readers more distinctly than the figures how the curve gradually changes its shape. For $r = 1$, it is plainly logarithmic; for $r = 2$, it appears practically of the same shape, except that the first time unit shows no deaths. Even for $r = 6$, the general shape of the curve resembles the first one except that it is flattened. For a while, the largest number of deaths occurs in the first unit of time in which any death takes place at all. However, at $r = 9$, the number of deaths in the first and second dying period are equal, and for $r > 9$, more organisms die in the second than in the first time unit. That this change occurs at $r = 9$, is, of course, the result of our choice of $q = 0.9$; but regardless of how we chose q, there will always be a r-limit beyond which the death in the first time unit is smaller than in the second. The maximum of the death rate shifts to third place for $r = 2 \times 9$, and to fourth place for $r = 3 \times 9$.

TABLE III

A Theoretical Case of Disinfection

Time n	r = 1	r = 2	r = 3	r = 4	r = 5	r = 6	r = 7	r = 8	r = 9	r = 10	r = 11	r = 12
	Survivors											
0	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000 000
1	100,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000 000
2	10,000	190,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
3	1,000	28,000	271,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
4	100	3,700	52,300	343,900	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
5	10	460	8,560	81,460	409,510	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
6	1	55	1,270	15,850	114,265	468,559	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
7	0	6	176	2,728	25,691	149,693	521,703	1,000,000	1,000,000	1,000,000	1,000,000	1,000,000
8	0	1	23	432	5,024	38,092	186,894	569,533	1,000,000	1,000,000	1,000,000	1,000,000
9	0	0	3	64	891	9,331	52,972	225,159	612,580	1,000,000	1,000,000	1,000,000
10	0	0	0	8	147	1,634	12,794	70,190	225,159	651,322	1,000,000	1,000,000
11	0	0	0	1	23	396	2,749	18,533	52,972	263,901	686,189	1,000,000
12	0	0	0	0	4	50	541	4,329	8,331	70,191	302,643	717,570
13	0	0	0	0	1	7	99	920	891	12,795	89,562	340,997
14	0	0	0	0	0	1	13	180	64	1,635	18,535	110,870
15	0	0	0	0	0	0	3	34	3	147	2,751	25,637
16							0	6	0	9	295	4,330
17							0	1	0	0	24	541
18							0	0	0	0	2	50
19							0	0	0	0	0	3
20							0	0	0	0	0	0

Cells Dying per Unit Time

1	900,000	0	0	0	0	0	0	0	0	0	0	0
2	90,000	810,000	0	0	0	0	0	0	0	0	0	0
3	9,000	162,000	729,000	0	0	0	0	0	0	0	0	0
4	900	24,300	218,700	656,100	0	0	0	0	0	0	0	0
5	90	3,240	43,740	262,440	590,490	0	0	0	0	0	0	0
6	9	405	7,290	65,610	295,245	531,441	0	0	0	0	0	0
7	–	59	1,094	13,124	88,566	318,866	478,297	0	0	0	0	0
8	–	7	153	2,294	20,665	111,600	334,808	430,467	0	0	0	0
9	–	1	20	367	4,133	29,760	133,923	344,373	387,421	0	0	0
10	–	–	3	55	744	6,697	40,178	154,969	387,421	348,679	0	0
11	–	–	–	8	124	1,338	10,046	51,658	172,187	387,421	313,811	0
12	–	–	–	1	20	245	2,908	14,204	44,641	193,710	383,546	282,430
13	–	–	–	–	3	42	441	3,408	7,440	57,396	213,081	376,573
14	–	–	–	–	–	7	82	740	826	11,160	71,027	230,128
15	–	–	–	–	–	1	4	148	61	1,488	15,784	85,232
16							3	28	3	138	2,455	21,308
17							–	5	–	8	272	3,788
18							–	–	–	–	22	491
19							–	–	–	–	1	47
20							–	–	–	–	–	3

With increasing r, this block curve becomes more and more symmetrical.

To prove this point, the survivors for $r = 100$ molecules were computed. These figures show a type of curve absolutely different from

FIG. 5. Survivor curves and deaths per unit time for different numbers of reacting molecules per cell

that for $r = 1$; it resembles, to the author's unprejudiced mind, a variability curve, and with this curve before one's eyes, it is difficult to realize that it represents the order of death of absolutely uniform organisms which have all exactly the same resistance. This order of death is brought about by the circumstance that with 100 molecules, the reaction is not yet uniform in all cells, and it is the law of chance that still rules.

Just as conspicuous as the gradual change of shape of the black curves of deaths per unit time is the fact that for any r larger than 1,

TABLE IV

The Case of 100 Reacting Molecules in the "Theoretical Case of Disinfection"

Time	Survivors	Dying per unit time	Time	Survivors	Dying per unit time
1–99	1,000,000	0	115	39,827	32,629
100	999,973	27	116	20,567	19,260
101	999,678	295	117	9,993	10,574
102	998,055	1,623	118	4,575	5,418
103	992,163	4,892	119	1,977	2,598
104	976,288	15,875	120	807	1,170
105	942,422	33,866	121	312	495
106	882,643	59,779	122	114	198
107	793,748	88,895	123	40	74
108	678,925	114,823	124	14	26
109	548,509	130,416	125	5	9
110	416,644	131,865	126	2	3
111	296,767	119,877	127	1	1
112	197,979	98,788			
113	123,677	74,302			
114	72,456	51,221			

there is no death at all for a certain time; this time increases in direct ratio with r. We must expect this; if a large number of molecules must be destroyed before the cell has lost the power of recovery, it is very improbable that all the molecules in one cell will be destroyed in the first time unit. This improbability increases with the number of reacting molecules. If, however, only one molecule per cell exists, the largest number of deaths in the first unit of time is unavoidable.

The dominance of the logarithmic order of death with bacteria seems to indicate that they contain, among others, one peculiar molecule

extremely sensitive to heat whose inactivation prevents any further multiplication; or there may be several such molecules, but the inactivation of any one of them means death. In many instances, however, the death rate $\frac{1}{t} ln \frac{a}{b}$ is not constant, but decreases. In a few cases it increases. It becomes necessary to study the meaning of this, and to compute the death rates for the theoretical cases present in Table III.

The results in Table V show that the death rate computed as in ordinary disinfection experiments is distinctly increasing in all cases

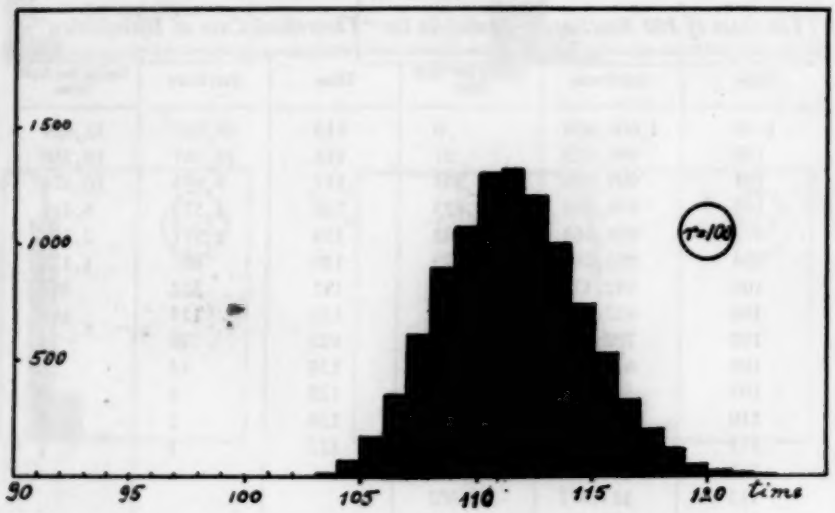

FIG. 6. Deaths per unit time with 100 reacting molecules per cell

except for $r = 1$, and the increase is very noticeable even if we chose as zero time, n_0, not the beginning of the exposure, but the beginning of dying. The larger r, the larger is the increase. This becomes more conspicious if we consider the *relative* "death rates," taking the rate of the first time unit = 100.

As a matter of fact, an increasing death rate has been very rarely observed. Reichenbach's (1911) unknown sporeformer, Chick's Staphylococcus and Myers' Bacillus 25 are probably the only cases on record for an increase of K. If the death rate is not constant, it

usually decreases. The decrease can be easily accounted for by the assumption of a graded resistance, the mass law holding for each grade. The death rate is at first influenced by the less resistant cells, and towards the end of the experiment, when the more sensitive cells are all dead, the resistant survivors show a lower death rate.

This circumstance that the death rate is either constant or decreasing, but very rarely increasing, is another strong argument for the assumption of $r = 1$ in bacteria.

TABLE V

"Death Rates" of the "Theoretical Case of Disinfection"

$$K = \frac{1}{t} \log \frac{a}{b}$$

Time interval	$r = 1$	$r = 2$		$r = 3$		$r = 4$		$r = 10$		
	$t_0 = 0$	$t_0 = 0$	$t_0 = 1$	$t_0 = 0$	$t_0 = 2$	$t_0 = 0$	$t_0 = 3$	Time	$t_0 = 0$	$t_0 = 9$
0–1	1.0	0	—	0	—	0	—	9–10	0.0213	0.213
1–2	1.0	0.361	0.721	0	—	0	—	10–11	0.0526	0.289
2–3	1.0	0.518	0.776	0.189	0.567	0	—	11–12	0.0961	0.384
3–4	1.0	0.608	0.811	0.320	0.641	0.116	0.453	12–13	0.1460	0.473
4–5	1.0	0.667	0.834	0.417	0.689	0.218	0.545	13–14	0.2000	0.557
5–6	1.0	0.706	0.852	0.483	0.724	0.300	0.600	14–15	0.2555	0.639
6–7	—	0.742	0.866	0.537	0.751	0.366	0.641	—	—	—
7–8	—	—	—	0.560	0.773	0.421	0.673	—	—	—
8–9	—	—	—	—	—	0.466	0.699	—	—	—
Relative Death Rates										
	100		100		100		100			100
	100		108		113		117			136
	100		112		122		130			180
	100		116		128		138			222
	100		118		133		146			262
	100		120		136		151			300

The question may well be asked how the type of curve illustrated in Figs. 5 and 6 will ever come to show that all large organisms behave alike and die at the same moment. The explanation for this involves the time factor.

In the development of the formula upon which this discussion is based, it was implied that, when we had two reacting molecules per

unit, the units were twice as large. Otherwise, it would mean a change of concentration. But in computing Tables III and IV, the same initial number of cells, $a = 1{,}000{,}000$, has been used. The 1,000,000 cells for $r = 12$ contain 12 times as many reacting molecules as the 1,000,000 cells for $r = 1$. This has not been important for our considerations so far, because neither the shapes of the curves nor the death rates would be changed at all if for $r = 2$, the initial number of cells were chosen as 500,000.

But if in one case, we have 10 times as many molecules as in the other, it is certain that it will take more time to kill all the organisms containing each 10 times as many molecules. Our Tables III and IV show this very distinctly. Since the survivor curve approaches zero asymptotically, we might best compare the times required to reduce the number of living cells to less than 1. This requires for $r = 1$ only 6 minutes, for $r = 4$ about 12 minutes, for $r = 11$ about 18 minutes, and for $r = 100$ about 128 minutes. Of this time, a considerable portion passes before any deaths occur at all. This time increases with the number of reacting molecules. It increases not only absolutely, but also relatively. Computing the time of action without death in terms of the total time needed to kill 99.9999 per cent of all organisms, we obtain:

TABLE VI

Time Required to Kill 999,999 Out of 1,000,000 Cells

r	Total time for disinfection	Time before first death		Period of dying	
		Absolute	Per cent of total time	Absolute	Per cent of total time
1	6	0	0	6	100
2	8	1	13	7	87
3	10	2	20	8	80
4	12	3	27	8	73
6	14	5	36	9	64
8	16	7	41	10	59
10	17	9	53	8	47
12	20	11	55	9	45
100	128	99	77	29	23

If the curves of the deaths per unit time are plotted so that the total time required for killing all cells is reduced to the same scale, we obtain

the pictures represented in Fig. 7. The total abscissa presented, 6 time units, is required to reduce 1,000,000 living cells to less than one; this cannot be shown in the curves because 1,000 cells are the smallest number that is barely visible on this scale. The figure illustrates,

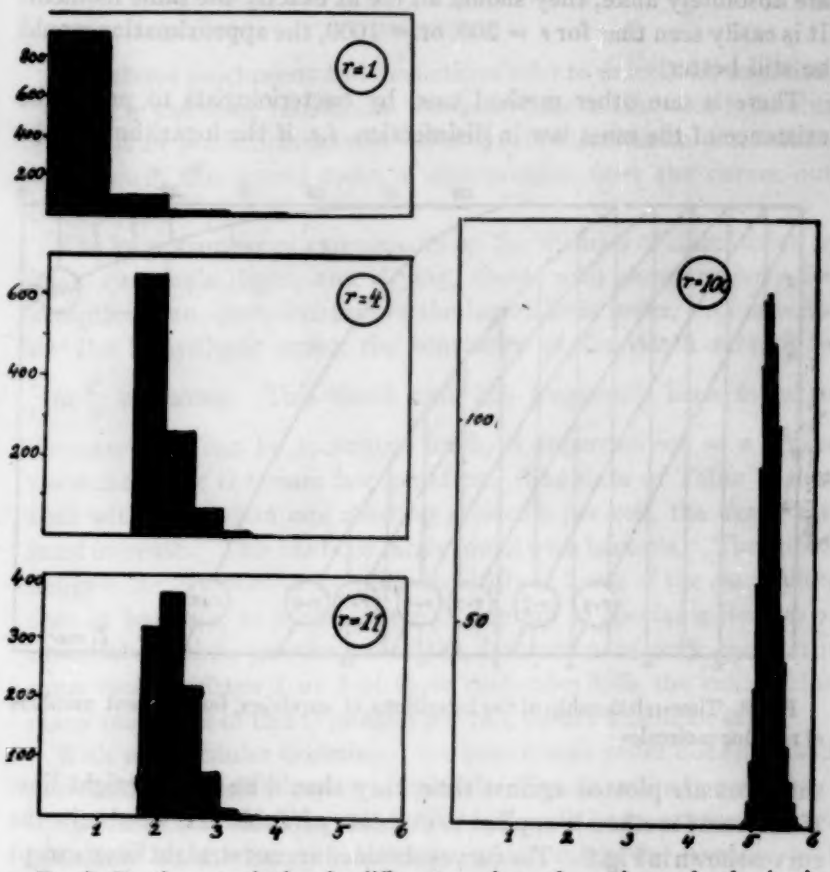

FIG. 7. Deaths per unit time for different numbers of reacting molecules (as in Figs. 5 and 6), but drawn to a standard time scale

however, how, with increasing r, the relative time required for killing becomes less, and how, for $r = 100$, it approaches a line. It is not very likely that such a curve could be obtained by experimental measurement. If we measured even every half minute, we would

find no cells killed until the 9th half minute, 23,000 killed in the 10th half, 947,000 in the 11th half, and 20,000 in the last half. This means practically all cells, (94.7 per cent) killed between 5 and 5.5 minutes. This is a good approximation to the "vitalists'" claim that if all cells are absolutely alike, they should all die at exactly the same moment. It is easily seen that for $r = 200$, or $= 1000$, the approximation would be still better.

There is one other method used by bacteriologists to prove the existence of the mass law in disinfection, *i.e.* if the logarithms of the

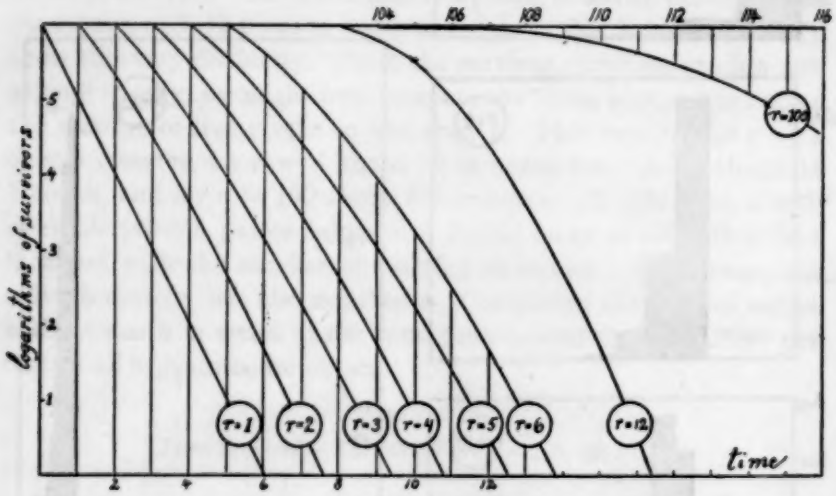

FIG. 8. Time-relationship of the logarithms of survivors, for different numbers of reacting molecules

survivors are plotted against time, they should be on a straight line. If this same method is applied to our data of Table III, we obtain the curves shown in Fig. 8. The curves obtained are not straight lines (except for $r = 1$) but they are near enough to straight lines to be considered as such if we omit the initial number. Unfortunately, many bacteriologists have disregarded the initial number and have started the counting of survivors after the bacteria had been exposed to the unfavorable condition for some time. All data by Paul and his associates on the action of acids upon Staphylococcus, and many data by Chick are of no value on account of this omission. Any reconstruc-

tion or extrapolation would be quite arbitrary. This circumstance reduces the number of proofs for the logarithmic order considerably. If the initial number is included, a straight-line relationship of the survivors is a fairly good criterion for the logarithmic order.

DISCUSSION

The above conclusions and deductions refer to unicellular organisms of exactly the same resistance. No allowance is made for individual variation or graded resistance. If a chance distribution of resistance is assumed, this would mean a superposition over the curves outlined above.

The large number of experiments on the theories of disinfection by heat, chemicals, light, and drying, show, with comparatively few exceptions, an approximation to the logarithmic order. As criterion for the logarithmic order, the constancy of the death rate, $K = \frac{1}{t} ln \frac{a}{b}$ is chosen. This death rate has frequently been found to decrease; this can be accounted for by a superposition of a graded resistance over the mass law equation. The data of Table V show that with more than one reacting molecule per cell, the death rate must increase. This has been rarely found with bacteria. The author believes the evidence to be quite decidedly in favor of the assumption that in bacteria, as a rule, there is a group of special molecules so essential for their existence that the destruction of only one and in some cases perhaps 2 or 3 of these molecules kills the cells. How many molecules of this type exist per cell, we are unable to say.

With multicellular organisms, the logarithmic order does not hold, nor could we possibly expect it to hold. The situation becomes very complicated. Even supposing that all cells are exposed simultaneously to the harmful influence which might be possible with exposure to heat, but not to chemicals, death of the entire organism will begin with death of the individual cells. But the death of one cell does not mean the death of the entire organism. We have here a repetition of the relation between molecules and cells on a higher level; this time, death depends upon the number of cells that must be killed to prevent recovery of the organism. The mathematical treatment of this problem seems possible, but extremely complex.

CONCLUSIONS

Death of unicellular organisms is brought about by the inactivation of a certain number of essential molecules in the cell.

If the number of these essential molecules is only one per cell, the order of death is the same as if the cell were identical with this molecule; the order of death is logarithmic following the mass law.

If more than one molecule must be inactivated before the cell dies, the order of death is not logarithmic. With 2 or 3 molecules, it still resembles the logarithmic order, but with an increasing number of reacting molecules, it approaches more and more the order of death known with higher organisms, namely a period of no death, followed by a comparatively short period of rapid death.

The decision whether or not the logarithmic order exists, should be based upon the constancy of the death rate $K = \frac{1}{t} ln \frac{a}{a-x}$. The existence of a straight line when logarithms of survivors are plotted against time, is not sufficient proof unless the initial number of cells is included.

These deductions are made with the assumption that all organisms are exactly alike, and show no individual variations or graded resistance.

With most bacteria, the order of death is so nearly logarithmic that death must be brought about by the inactivation of only one molecule, though there may be several molecules of this same type in each cell.

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DISSIMILARITY OF INNER AND OUTER PROTOPLASMIC SURFACES IN VALONIA. II

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In an earlier publication[1] from this laboratory experiments have been described in which the P.D. was measured between the inner and outer protoplasmic surfaces of *Valonia macrophysa*.

Electrical connection with the sap of the (very large) vacuole was made by piercing the cell with a fine glass capillary tube filled with artificial sap. It was found that on standing the protoplasm becomes attached to the glass, forming a seal which prevents short circuit between the cell wall and the sap at the spot where the tube passes through the protoplasm. When both the inner and outer surfaces of the protoplasm were in contact with approximately the same solution (natural sap inside and natural or artificial sap outside) a P.D. of from 4 to 38 mv. was observed, the inner surface being positive to the outer. The average of 100 readings was 14.5 mv. An observed P.D. of this magnitude in the apparently symmetrical chain: sap | protoplasm | sap, led to the conclusion that the protoplasm itself is not symmetrical. As a working hypothesis it has been assumed that the protoplasm is made up of three layers, an outer, non-aqueous layer, X, the aqueous main body of the protoplasm, W, and an inner, non-aqueous layer, Y, different from X.

In these experiments, however, we failed to appreciate the effect of sea water wetting a large fraction of the surface of the cell when we measured the P.D. against a second solution applied at one end only. From further experiments with improved technique we have found that the value reported for the P.D. of this system is too low, and also that the P.D. undergoes characteristic changes with time. These new data support the conclusions as to the asymmetric structure of protoplasm which were advanced in the earlier paper.

[1] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, **11**, 193.

In nearly all our earlier measurements, the *Valonia* cell hung free from the capillary tube on which it was impaled; in a few cases the cell was also supported from below on a glass ring. In all cases, whether the contact was stationary or flowing, the artificial sap or other external solution was applied to the lower tip of the cell only, while the rest of the surface remained wet with sea water, or with

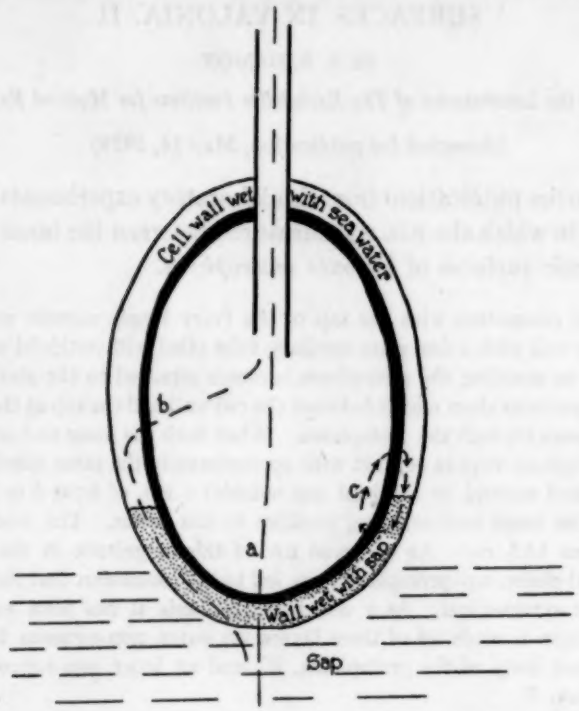

FIG. 1. Diagram of a cell of *Valonia macrophysa* impaled on a capillary, showing conditions when the cell is in contact with more than one solution. At *a* is shown the path along which we desire to measure the P.D.; at *b* is a parallel path in which the E.M.F. across the protoplasm is lower. Differences in the E.M.F. across the protoplasm lead to local currents as illustrated at *c*.

a mixture of sea water and the new solution. Under such conditions we should expect a different E.M.F. across the protoplasm at the upper part of the cell, where the cell wall is imbibed with sea water, from the E.M.F. at the tip, where the protoplasm is in contact with some other solution, such as sap. The observed P.D. will have an intermediate value depending on the relative areas wet by the two solutions. The reason for this is that in addition to the desired path at *a* (Fig. 1),

there are parallel paths, such as *b*, where the E.M.F. is lower. Hence there will be local currents in the direction as represented by the arrows in the path *c*, which will tend to decrease the P.D. against sap, and possibly to increase that against sea water.

These circuits may in a general way be compared to a battery of galvanic cells connected in parallel, where some of the cells are of higher E.M.F. (corresponding to the parts of the protoplasm where sap is applied), and others are of lower E.M.F. (corresponding to the parts of the protoplasm in contact with sea water). Increasing the area in contact with sap is comparable to adding cells of the higher E.M.F., and simultaneously cutting out the same number of cells of the lower E.M.F.

These views were confirmed by experiments in which the P.D. was measured (1) with only the tip of the cell dipping into artificial sap, while the greater part of the surface of the cell remained wet with sea water, and (2) with the entire cell immersed in artificial sap. The following is a typical result:

Tip only in artificial sap, cell wall wet with sea water.....	8.5 mv.
Entire cell immersed in artificial sap, 2 minutes later.....	32.6 mv.
Tip only immersed, but cell wall wet with artificial sap, 1 minute later.....	30.9 mv.

Evidently the P.D. against a given solution applied at one end of the cell may be greatly affected by the presence of a different solution wetting other parts of the cell. Additional evidence of this will be presented later.

In view of this fact, it seemed necessary to carry out further measurements of P.D. in such a way that solutions applied to the cell should wet the entire surface. Also, to prevent contamination by salts leached out of the cell wall, etc., it seemed desirable to apply the solutions by means of a flowing contact such that a steady stream should flow continuously over the entire cell. Our former method of impaling the cell from above was not suited to this procedure, since liquids running down over the cell would be liable to pull it away from the capillary tube, breaking the seal. A better way is to impale the cell on top of a vertical capillary pointing upward; the solutions can then be led on at the top of the cell. Cells so impaled, however, must be supported in such a way that the point of the capillary cannot scratch the protoplasm. This is done conveniently by means of a cork mount as shown in Fig. 2.

The lower half of the singly bored cork stopper fits tightly over the glass tube, just below the tip where the tube is drawn out to a capillary. The upper half of the cork is cut away, leaving four prongs, on or in which the cell rests when it has been impaled on the capillary. The complete cell holder, Fig. 3, is a modification of the holder described in our earlier paper. The 250-cc. wide-mouth bottle, *C*, is fitted with a two-hole rubber stopper, through which pass the tube on which the cell is impaled, and the funnel tube, *H*. The bottle and tubes are filled with artificial *Valonia* sap. When the cell is impaled, pressure is applied by blowing

in at *H*, so that the cell is kept turgid while it is pressed down on or within the prongs of the cork mount. After the cell is once in place, the hydrostatic pressure (5 or 6 inches) from the level of solution in *H* is sufficient to keep the cell distended. Before the impaled cells are used, they are allowed to stand immersed in sea water for two days or longer, to see whether signs of injury appear, and to permit the formation of a good seal.

In measuring P.D. across the protoplasm, contact with the inside of the cell is made through the funnel, *H*. A string wet with artificial sap leads from *H* to a beaker of artificial sap into which dips the siphon of a saturated KCl-calomel

FIG. 2. Photograph showing how the impaled cell is supported by a cork mount.

electrode. Contact with the outside of the cell is made through a strip of wet filter paper touching the highest point of the cell. The solutions applied flow down the filter paper and over the entire surface of the cell. The apparatus used for holding the filter paper is shown in Fig. 3.

The cell is supported over the funnel, *F*, which catches waste solution and carries it off to the drain. A light platform of paraffin-coated wood resting on the rim of the funnel carries glass rails, *r* and *r'*, for supporting the strip of filter paper, *p*, a glass adapter-shaped tube to guide drip from the filter paper well away from the cell, and a cup, *c*, in which connection is made between the filter paper and the

siphon from a second saturated KCl-calomel electrode. The cup, *c*, has a hole drilled in the side, just under the platform, through which waste solution overflows and drips into the funnel. Solutions from reservoir bottles, flowing down along short lengths of string, *s* and *s'*, are led on the filter paper at the points where it passes over the glass rails, so that a slow stream flows down the paper on both sides. The solution to be applied to the cell is led on the filter paper at *r'*, and molal KCl solution at *r;* contact between the calomel electrode and the solution applied to the cell is thus made through a flowing junction, which should be

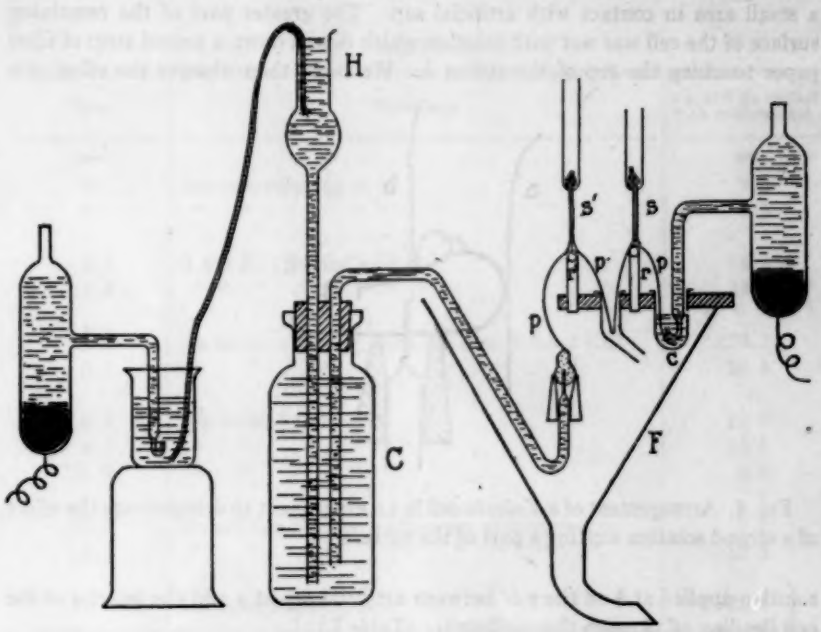

FIG. 3. Apparatus used in measuring the P.D. across the protoplasm of a *Valonia* cell impaled on a capillary filled with artificial sap. The solution applied externally is led on at the top of the cell and flows down over the entire surface.

reproducible, and quickly established when solutions are changed. An advantage of this arrangement is that solutions are changed without opening the circuit, permitting us to obtain an unbroken record of changes in P.D.

Measurements of P.D. reported in this paper, as in the preceding article, were made by means of a Compton quadrant electrometer.

The following experiment, using the new technique of impaling, demonstrates the effect of a second solution wetting a part of the

surface of the cell. The P.D. between the inside of the cell and artificial sap applied to a small spot at one end was measured while most of the remaining surface was wet with a stream of sea water, and again after the sea water had been replaced by a stream of 0.6 M KCl solution.

A rather long cell was impaled as shown in Fig. 4, in such a way that it rested with the long axis nearly horizontal. A strip of filter paper, down which flowed a stream of artificial sap, made contact with one end of the cell at *a*, but brought only a small area in contact with artificial sap. The greater part of the remaining surface of the cell was wet with solution which flowed down a second strip of filter paper touching the top of the cell at *b*. We could then observe the effect of a

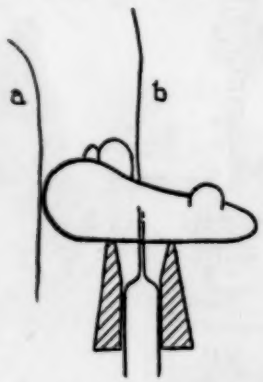

FIG. 4. Arrangement of a *Valonia* cell in an experiment to demonstrate the effect of a second solution wetting a part of the surface.

solution applied at *b* on the P.D. between artificial sap at *a* and the interior of the cell (leading off through the capillary). (Table I.)

As in our earlier paper, the sign of the P.D. is that of the inside of the cell. A positive potential means that the inside of the cell is more positive than the solution applied externally: *i.e.*, positive current tends to flow in the external circuit from the capillary through the electrometer to the solution bathing the outside of the cell.

Obviously, in measurements with *Valonia* (and probably with other marine algae) where the surface must be kept wet with highly conducting solutions, it is undesirable to use methods in which the surface is brought in contact with more than one solution at a time. Experiments of Osterhout and Harris[2] on *Nitella*, however, indicate that this

[2] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761.

criticism does not apply to measurements on fresh water algae where the surface of the cell is bathed with a solution of high resistance, such as tap water, making the electrical resistance of the cell wall so high, as compared with that of the desired current path, that shunts through the cell wall have little effect. With *Valonia*, it is not practicable to decrease the conductivity of the cell wall by leaching with distilled water, since, except within rather narrow limits, dilute

TABLE I

Time	Description	P.D. at *a* (in contact with artificial sap)
min.		mv.
0	Sea water flowing at *b*	9.0
1	" " " " "	8.5
2.5	0.6 M KCl flowing at *b*	24.2
3.5	" " " " " "	23.6
4.5	No solution flowing, surface wet with 0.6 M KCl	20.2
5.5	" " " " " " " " "	19.3
6.5	Sea water flowing at *b*	20.0
8.5	" " " " "	12.8
11.5	" " " " "	8.8
12.5	No solution flowing, surface wet with sea water	9.2
15.5	" " " " " " " "	12.4
17	0.6 M KCl flowing at *b*	22.0

solutions prove highly injurious, even when made isotonic with sea water by the addition of a suitable non-electrolyte such as sugar.

Although Osterhout has observed[3] that *Valonia* cells immersed in their own (natural or artificial) sap live but a short time, generally less than a week, we have found that they easily survive exposure to sap for periods of several hours. Using the apparatus described above, we have followed changes in P.D. against artificial sap applied externally in runs which lasted as long as ten hours.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1924–25, **7**, 561.

These measurements were carried out at Bermuda during the months of April and May, at room temperatures ranging from 19.5° to 24°C. In most cases, the temperature variation during a single run was less than 1°. In all measurements reported, the cells appeared in good condition at the end of the experiment. They were then returned to a sea water bath and watched for several days; in all cases they continued to look healthy, and gave with sea water the usual P.D. of 5 to 10 mv. Indeed, cells which had been exposed to artificial sap apparently lived longer than others impaled at the same time, and merely kept in sea water, but this may be explained by the fact that the best looking cells were chosen for measurement. The possibility that brief exposure to sap may be beneficial, however, is suggested by the observation of Dr. L. R. Blinks that addition of a small amount of KCl to the sea water in which cells of *Valonia* are immersed assists in keeping the cells alive under unfavorable conditions.

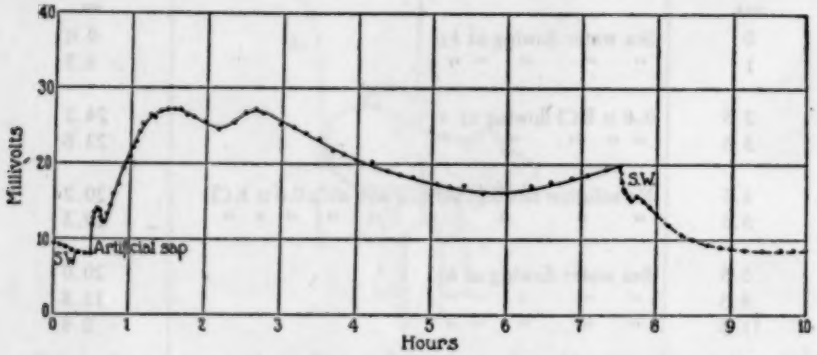

Fig. 5. Curve showing changes in P.D. across *Valonia* protoplasm when the outside of the cell is in contact with artificial sap, and also when the sap applied externally is replaced by sea water.

Results of our experiments are shown graphically in Figs. 5 to 8. It is apparent that the system is changing continuously during the measurement, and that there is accordingly no single value which may be assigned to the P.D. across *Valonia* protoplasm when both surfaces are in contact with *Valonia* sap. In most of the measurements reported here, the maximum P.D. observed was between 25 and 35 mv., but occasionally still higher values are found: an extreme case, when the P.D. rose to 81.8 mv., is shown in Fig. 7.

The graph, Fig. 5, has been selected as a fairly representative curve, but comparison with Fig. 6 (*A*, *B*, and *C*), Figs. 7, and 8*A*, shows that there is a considerable variation in the behavior of different cells. In general, however, we may say

that during the first 90 minutes the P.D. rises to a maximum, falls rapidly to a minimum (which, however, is higher than the P.D. with sea water), then rises slowly to a second maximum. After this, the behavior of different cells is too

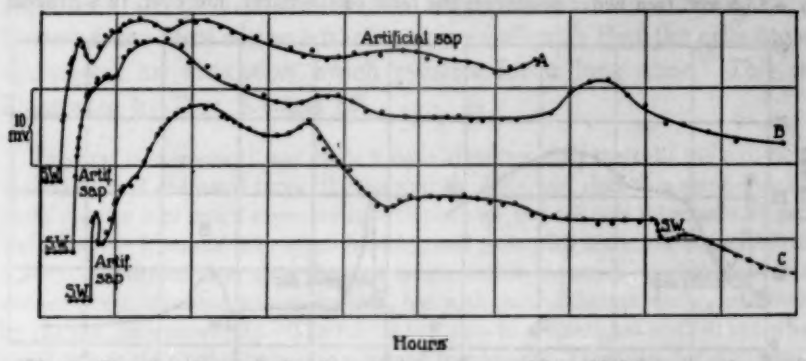

Fig. 6. Showing the variation in P.D.-time curves with artificial sap as observed with different cells. In order to prevent confusion the graphs are separated by vertical displacement (the value in sea water at the start being about 8 millivolts in each case).

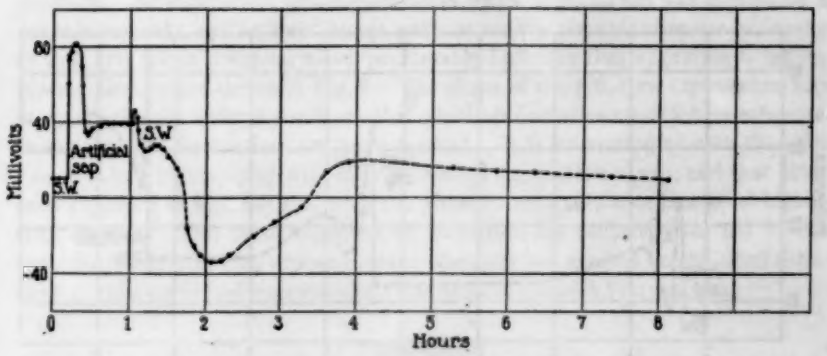

FIG. 7. P.D.-time curve with artificial sap, followed by sea water. An unusual case, in which the changes in P.D. are larger than commonly observed. The usual behavior is shown in Figs. 5 and 6.

varied to permit a single general statement. The first peak is commonly lower than the maximal P.D. observed later, but it may, as in Fig. 7, be much higher; the first and second maxima may be widely separated, as in Fig. 8*A*, or rarely, as in Fig. 6*B*, they may overlap, so that the curve does not pass through a minimum.

In Figs. 5, 6*C*, and 8*B* are shown also the changes in P.D. which are commonly

observed when the cells are returned to sea water (using flowing contact) after they have been exposed to artificial sap. In Fig. 7 is shown a case unusual in that the changes are much greater than those ordinarily found, the P.D. falling to −33.5 mv. (sea water positive); the form of the curve, however, in a general

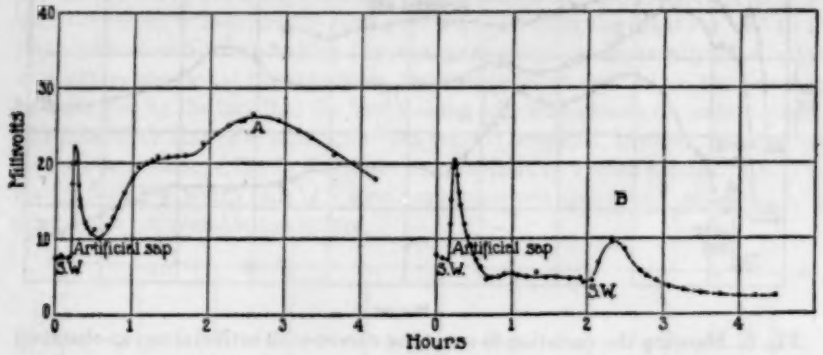

FIG. 8. P.D.-time curves with artificial sap, measured on the same cell, curve *B* fourteen days after curve *A*, showing the change in behavior caused by exposure to artificial sap.

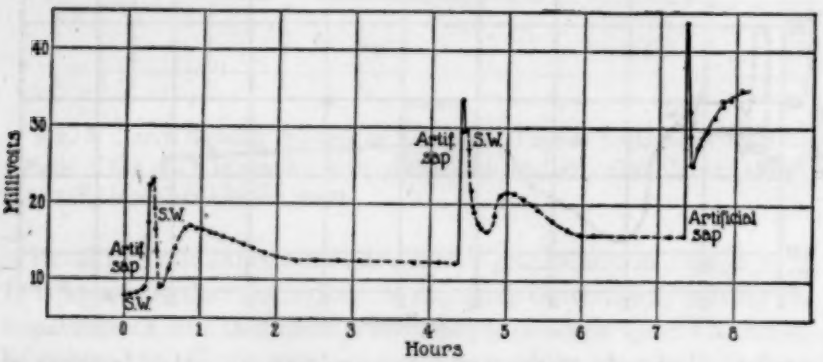

FIG. 9. Curve showing changes in P.D. when the cell is exposed alternately to artificial sap and to sea water, the treatment with artificial sap being too short to produce much alteration.

way resembles the usual one. In most cases, the P.D. at first falls fairly rapidly, passes through a minimum, rises again to a maximum (lower than the P.D. with sap), and then falls slowly and steadily. After a number of hours in sea water, the P.D. becomes more or less constant at a value between 5 and 10 mv., approximately the same as before the experiment.

The appearance of the cells several days after the experiment, and the P.D.'s which they give with sea water, indicate that no permanent injury has been received as a result of exposure to artificial sap. If such cells are used in the second measurement with artificial sap, however, the form of the P.D.-time curve indicates that the cells have undergone an alteration which persists for a long time. This is illustrated by Figs. 8*A* and 8*B*.

The first measurement was made 9 days after impaling the cell; the P.D.-time curve (*A*) is of the usual form: it rises sharply, falls, and rises to a second maximum. After four hours exposure to artificial sap, the cell was returned to a sea water bath. It continued to look healthy, and gave with sea water a P.D. of 7 to 8.5 mv. Fourteen days after the first measurement, a second measurement was carried out in the same way as the first, but with quite different results, as shown by the P.D.-time curve (*B*). The P.D. at first rose to a maximum and fell again as usual, but it then failed to rise to a second maximum. This behavior is typical. The cell was not permanently injured, since, returned to sea water, it continued to live and 10 days later gave with sea water a P.D. of 6.1 mv.

Since prolonged exposure to sap produces an irreversible (or very slowly reversible) change in the protoplasm, it is interesting to compare the effect of brief exposure. An experiment was carried out in which artificial sap was applied for a few minutes only, and replaced by sea water as soon as possible after the beginning of that first fall in potential which presumably indicates that alteration is taking place. Results are shown in Fig. 9. The shape of the P.D.-time curve when sap is replaced by sea water is similar to that which we find after much longer exposure to sap, but the fluctuations are more marked. It is interesting to note that the P.D. rises to a higher value with each successive application of sap, and that after each exposure to sap, the P.D. with sea water returns to a constant level higher than the last. This must indicate that alteration has occurred, but not to the same degree as after long exposure, since when sap was applied for the third time, the P.D.-time curve had approximately the usual shape with two maxima.

DISCUSSION

While it is too early to attempt an explanation of the cause of the P.D. across the protoplasm, it may be worth while to consider what sort of alteration might be expected to lead to changes in P.D. consistent with those which we have observed.

The alteration caused by artificial sap applied externally is probably of a very simple nature, since we can hardly expect the cells to survive a fundamental change in the structure of the protoplasm. Perhaps

the simplest hypothesis is that this alteration consists merely in an increased concentration of KCl in the main body of the protoplasm. The characteristic fluctuations in P.D. with artificial sap can be explained, at least qualitatively, as due to such an increase, on the basis of the theory of protoplasmic layers which Osterhout and Harris[4] have found useful in interpreting their results with *Nitella*.

This theory assumes that the protoplasm has an outer, non-aqueous surface layer (X) and an inner, non-aqueous surface layer (Y), different from (X), the two being separated by the aqueous main body of the protoplasm (W). The observed P.D. is then the algebraic sum of the P.D. across X, between the external solution and W, plus the P.D. across Y between W and the vacuolar sap. We assume that the E.M.F. at Y is opposite in sign to that at X and that both these E.M.F.'s are large as compared with their algebraic sum when the external solution is sea water. This assumption is supported, for the case of *Nitella*, by the changes in P.D. observed when the cells are killed by applying solutions saturated with chloroform (current of injury),[4] and for the case of *Valonia* by (unpublished) results of analogous experiments.

These ideas are illustrated by hypothetical diagrams, Fig. 10, where the direction in which positive current tends to flow is indicated by the direction of the arrows, and the relative magnitude of the E.M.F.'s by their length. The observed P.D., the resultant of the E.M.F.'s at X and Y, is shown by feathered arrows.

A represents conditions when the outside of the cell is bathed with sea water, B, immediately after the sea water outside the cell, and imbibed in the cell wall, had been replaced by artificial sap. That is, we suppose that the first sharp rise in P.D. occurs entirely at X: this is shown by lengthening the X arrow, and also the feathered arrow which represents the observed P.D. This change in P.D. is no doubt connected with the fact that KCl enters the cell more readily than NaCl, but no attempt is made here to explain the cause of these potentials. They may, for example, be phase-boundary potentials, or they may be diffusion potentials in the non-aqueous phase.

The initial concentration of K salt in W or X is probably low, since increasing the K concentration outside increases the P.D. at X. (If

[4] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673.

the K concentration in *W* were high, an increase in K outside, making conditions at the two surfaces of *X* more nearly alike, would be

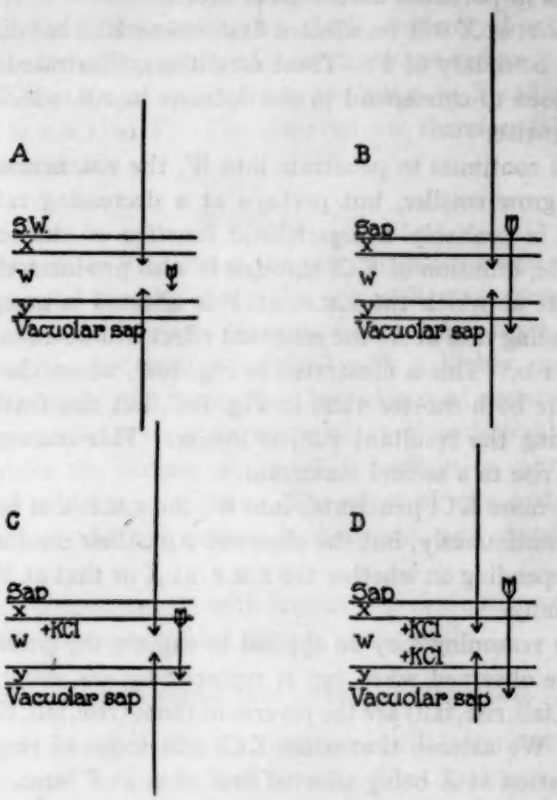

FIG. 10. Hypothetical diagram illustrating the theory of protoplasmic layers. The length of the arrows indicates the relative magnitude of E.M.F.'s assumed to exist across the inner and outer surface layers. The direction of the arrows is that in which positive current tends to flow. The observed resultant P.D. is shown by feathered arrows. *A* represents conditions when the external solution is sea water; *B*, conditions immediately after sea water has been replaced by artificial sap. *C* and *D* illustrate how the penetration of KCl into the protoplasm may cause first a decrease, and then an increase in the observed P.D. (The values represented by the lengths of arrows are fictitious, and do not correspond to any particular experiment.)

expected to decrease the P.D. across this layer.) Now, the entrance of KCl, increasing the concentration in *W*, will tend to make *W* more

nearly like sap, and thus by making conditions on the inner and outer surfaces of X and Y more nearly alike, will tend to decrease the differences in potential across these layers.

The E.M.F. at X will be affected first, before KCl has diffused across W to the boundary of Y. These conditions, illustrated in Fig. 10C, are supposed to correspond to the decrease in P.D. which follows the first sharp rise.

As KCl continues to penetrate into W, the P.D. across X will continue to grow smaller, but perhaps at a decreasing rate, since the potential is probably a logarithmic function of the concentration. Meanwhile, diffusion of KCl through W also produces changes at Y. If the rate at which the E.M.F. at Y is affected is greater than the corresponding rate at X, the resultant effect will be an increase in the observed P.D. This is illustrated in Fig. 10D, where the arrows at X and Y are both shorter than in Fig. 10C, but the feathered arrow, representing the resultant P.D., is longer. This corresponds to the observed rise to a second maximum.

As still more KCl penetrates into W, the E.M.F.'s at both X and Y will fall continuously, but the observed P.D., their resultant, may rise or fall, depending on whether the E.M.F. at X or that at Y changes the more rapidly.

Similar reasoning may be applied to explain the potential changes which are observed when sap is replaced by sea water, since these changes (fall, rise, fall) are the reverse of those (rise, fall, rise) produced by sap. We assume that some KCl comes out of protoplasm, the concentration at X being affected first, that at Y later.

The increase in P.D. observed when sea water and sap are applied alternately (Fig. 9), may be ascribed to a decrease in the E.M.F. at Y, produced by the penetration of a small amount of KCl. Changes at Y are less readily reversed than corresponding changes at X, because of the greater distance of Y from the outer surface.

It is probable, however, that after long exposure to sap a considerable concentration of KCl remains in W, even after the cell has been standing in sea water for several days, and that the absolute values of the E.M.F.'s at X and Y are accordingly much less than with unused cells, although the observed P.D. in sea water, their algebraic sum, may not be different from its usual value. When the cell is measured against sap for a second time, the increase in E.M.F. at X, and hence in

the observed P.D., may be as great as in the first measurement, although the absolute values of the E.M.F.'s at X and Y are much smaller. As KCl penetrates into W, the E.M.F. at X, and with it the observed P.D., will decrease as before. At Y, however, the concentration of KCl is already so great, and the E.M.F. so reduced, that the addition of KCl no longer can decrease the E.M.F. at Y sufficiently to offset the fall in E.M.F. at X. The observed P.D. therefore fails to rise to a second maximum.

SUMMARY

In measurements of P.D. across the protoplasm in single cells, the presence of parallel circuits along the cell wall may cause serious difficulty. This is particularly the case with marine algae, such as *Valonia*, where the cell wall is imbibed with a highly conducting solution (sea water), and hence has low electrical resistance. In potential measurements on such material, it is undesirable to use methods in which the surface of the cell is brought in contact with more than one solution at a time. The effect of a second solution wetting a part of the cell surface is discussed, and demonstrated by experiment.

From further measurements with improved technique, we find that the value previously reported for the P.D. of the chain

Valonia sap | *Valonia* protoplasm | *Valonia* sap

is too low, and also that the P.D. undergoes characteristic changes during experiments lasting several hours. The maximum P.D. observed is usually between 25 and 35 mv., but occasionally higher values (up to 82 mv.) are found.

The appearance of the cells several days after the experiment, and the P.D.'s which they give with sea water, indicate that no permanent injury has been received as a result of exposure to artificial sap. If such cells are used in a second measurement with artificial sap, however, the form of the P.D.-time curve indicates that the cells have undergone an alteration which persists for a long time.

On the basis of the theory of protoplasmic layers, an attempt has been made to explain the observed changes in P.D. with time, assuming that these changes are due to penetration of KCl into the main body of the protoplasm.

PROTOPLASMIC POTENTIALS IN HALICYSTIS

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(Accepted for publication, September 14, 1929)

I

The capillary technique used for *Valonia macrophysa*[1] is logically available for other large cells of similar structure. I have recently applied it with success to the closely related *V. ventricosa* of Florida[2] and to *Halicystis*[3] of Bermuda (long confused with *V. ventricosa*[4]). *Halicystis* is a multinucleate cell which superficially resembles *Valonia*, but differs markedly in details of morphology and in the constitution of the vacuolar sap.[5] Its study by the methods developed for *Valonia* is therefore of importance from a comparative standpoint, and its strikingly different behavior must be interpreted in any general bioelectrical theory.

II

Mechanically, *Halicystis* is distinguished by having a more elastic, extensible wall than *Valonia*. The cells are not firm and hard but rather resilient to the touch. They are capable of more shrinkage and swelling without injury to the protoplasm. The tendency to shrink makes the cells more difficult to impale; most of the sap may be lost by spurting through the opening around the capillary before the wound closes. Neither may this loss be made up by sap from the capillary, since hydrostatic pressure through the latter causes a sufficient stream to wash away protoplasm opposite its opening. By the use of sharpened capillaries, however, and by twisting the cells slightly as they are pushed on, most of them promptly form a seal. They then live as long as two or three weeks thus impaled, resting upon cut corks as described by Damon. They may shrink noticeably in

[1] Damon, E. B., *J. Gen. Physiol.*, 1929–30, **13**, 207.

[2] Blinks, L. R., *Carnegie Inst. Washington Year Book No. 28*, 1928–29, in press.

[3] Probably *H. ovalis*. A descriptive paper on this organism is in preparation.

[4] Blinks, L. R., *Science*, 1927, **65**, 429.

[5] Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, **68**, 164.

size during this time, apparently by loss of sap into the capillary. Since the sap of *Halicystis* is less dense than sea water, the larger cells tend to float, and are easily dislodged from the capillary by sudden jar. Cells having a bit of the substrate (calcareous *Lithothamnion*) still adhering to the holdfast remain more surely seated. The most conveniently handled cells are those about 1 cm. in diameter. The capillary may not be pushed far enough into smaller cells to ensure a firm seal, and larger cells tend to collapse on impalement.

The capillaries, drawn on the ends of quarter inch glass tubing, are usually about 0.5 mm. in outside diameter, and from 0.5 to 1.0 cm. in length. They project into the vacuole of the cell 2 or 3 mm. They, and the bottles into which they connect, are filled with artificial *Halicystis* sap made to correspond to the analyses previously published. (The sap of the small cells used in these experiments was

TABLE I

Molar Composition of Saps Expressed as Per Cent of Halide

	A	B	C	D	E
	Sea water Bermuda	*Halicystis* Bermuda	*V. macrophysa* Bermuda	*V. macrophysa* Tortugas	*V. tenticosa* Tortugas
Cl + Br	100.00	100.00	100.00	100.00	100.00
K	2.15	2.58	86.24	82.33	94.74
Na	85.87	92.80	15.08	18.55	5.73
Ca	2.05	1.36	0.288	0.02	Trace
Mg	9.74	2.49	Trace ?	0.08	Trace
SO_4	6.26	Trace ?	Trace ?	0.04	Trace

Analyses: B by Dorcas.
A, C by Van der Pyl.
D, E by Cooper.

essentially the same in composition as that of the large floating cells, according to analysis by Mr. Jacques.) Sodium, potassium, and calcium chlorides are present about as in sea water, with magnesium less concentrated and sulfate absent. (Cells which have formed zoospores and recovered may show sulfate.) The sap thus differs strikingly from that of the *Valonias*, as shown in Table I.[5]

Connection to the outside of the cell was made according to Damon's method,[1] the desired solution flowing down a strip of filter paper in contact with the top of the cell. Certain measurements were likewise made with the cell completely immersed in solution. These P.D.'s were essentially the same as with a flowing contact. Wet strings or salt-agar bridges formed the connection to lead chloride or calomel electrodes. The measuring instruments were a Compton electrometer, and a calibrated vacuum-tube electrometer.

III

Immediately on impalement, the cells of *Halicystis* showed almost no P.D. across the protoplasm. This was the case no matter what solution the cell was bathed in: sea water, artificial sap, or various single salt solutions. The impalement probably caused temporary injury.

On standing in sea water, however, the impaled cells soon displayed a larger and larger P.D. Within 1 hour the value might reach 30 to 40 millivolts, with the *outside positive* to the measuring instrument (*i.e.* positive current tending to flow from outside through the electrometer to the vacuole by way of the capillary; thus the positive current if allowed to flow would be across the protoplasm from inner surface to outer surface). Then more slowly the P.D. continued to rise, usually reaching in a day a maximum which was maintained more or less steadily for as long as two weeks. The highest P.D. found under any condition was 90 millivolts, the lowest steady value in sea water about 50 millivolts. The average value for some 50 cells measured was about 70 millivolts. There was often a fluctuation between 60 and 80 millivolts with the same cell from time to time. All of these were in the direction *outside positive*, and it was not possible to reverse the P.D. by any treatment so far administered.

Exposure of the cells to concentrated and dilute sea water had little of the expected effect on the P.D. A drop of about 10 millivolts was produced by $\frac{1}{3}$ sea water (made isotonic by glycerine) but in one case of long exposure the P.D. returned and rose above the original value; $\frac{3}{4}$ sea water produced a variation of 5 millivolts without permanent effect.

On the contrary, solutions of each of the more important salt constituents of sea water produced an immediate effect, abolishing the P.D. completely. 0.6 M NaCl, 0.6 M KCl, 0.4 M $CaCl_2$, 0.6 M $MgSO_4$, 0.4 M $MgCl_2$, each caused the P.D. to fall to zero in a minute or two, and to remain zero during the exposure. There was occasionally a slight rise just after the preliminary fall, amounting to 5 to 10 millivolts and lasting 2 or 3 minutes (see Fig. 1).

Except $CaCl_2$, which is quite toxic, these solutions did not cause permanent alteration of the cells, even by exposures up to $\frac{1}{2}$ hour.

The P.D. was restored remarkably soon upon re-exposure to sea water. This recovery is shown in Fig. 1 for a typical cell, after exposure to 0.6 M KCl. The recovery was delayed after long exposures but was rapid when once initiated. There was characteristically an "over-shooting" by which the P.D. went to a higher level than before treatment, and

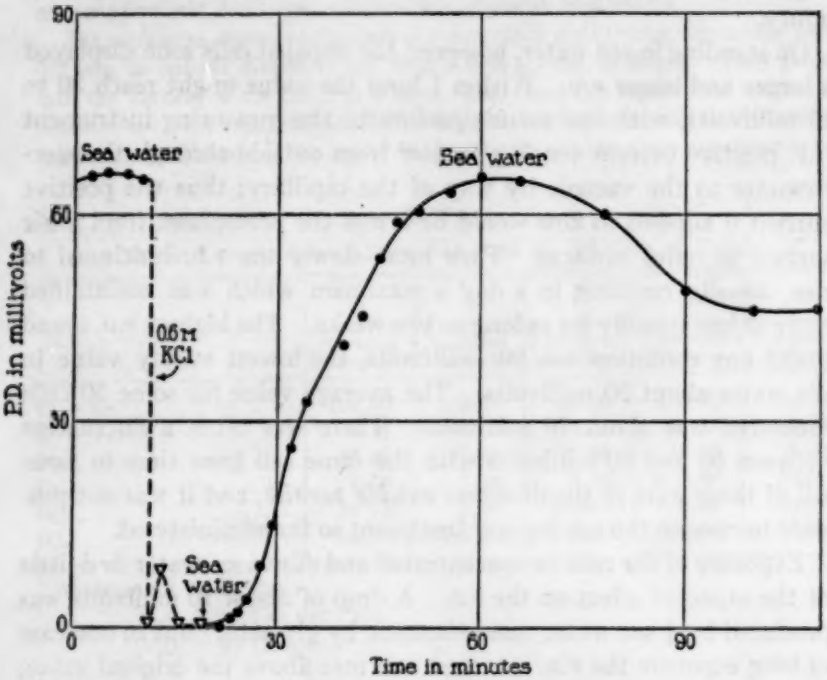

FIG. 1. P.D. in millivolts of an impaled cell of *Halicystis* exposed to 0.6 M KCl; with subsequent recovery in sea water. Time in minutes.

then descended slowly in 1 or 2 hours to a steady value. Occasionally there was only a partial recovery, quickly followed by death.

That the value was dependent on a balanced solution is evident from experiments with mixed salts. Thus the P.D. did not drop to zero when artificial sap was applied to the exterior of the cell, but remained for a long time at about 35 millivolts (Fig. 2). In one case recovery to over 60 millivolts occurred during such exposure. Injury ensued in another experiment, with disappearance of P.D. It is evident that

here again is an example of radial asymmetry in the protoplasm, since similar solutions applied to both sides of the protoplasm still may produce a high P.D. There is apparently a delicate balance at about this composition, since the value with sap is variable.

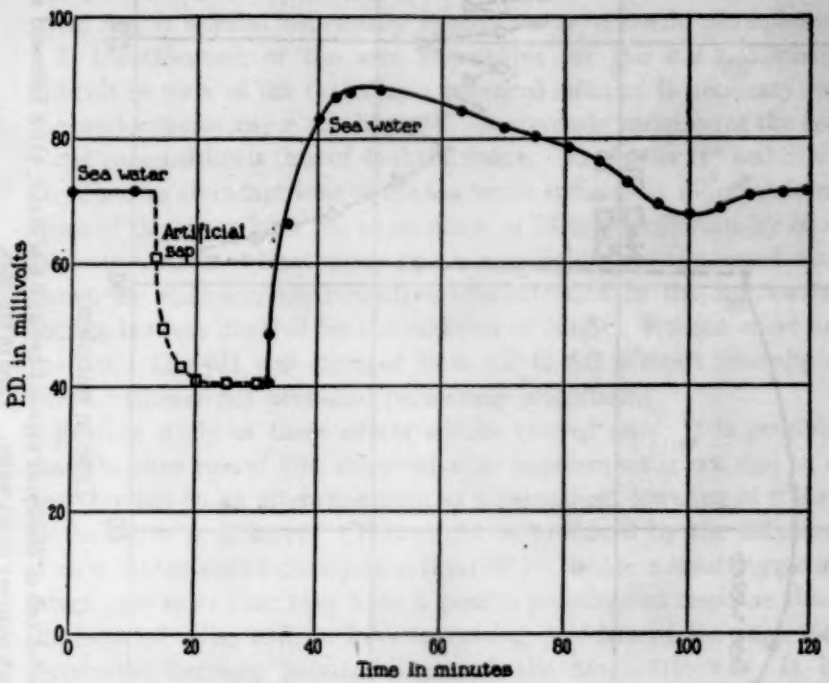

FIG. 2. P.D. in millivolts of an impaled cell of *Halicystis* exposed to artificial sap; with subsequent recovery in sea water. Time in minutes.

The P.D. was still less stable in simpler mixtures, as was exemplified by cells exposed to 0.6 M NaCl, 97.5 parts, + 0.4 M $CaCl_2$, 2.5 parts. The graph of Fig. 3 shows the course of P.D. variation in a cell exposed to this mixture. There is striking evidence here of alternate breakdown and recovery, which suggests the balance of processes dependent not on a single salt, but on several in proper proportion.

IV

It is not possible to draw full theoretical conclusions from the data so far available for *Halicystis*. Two striking facts stand out distinguishing it from *Valonia*.

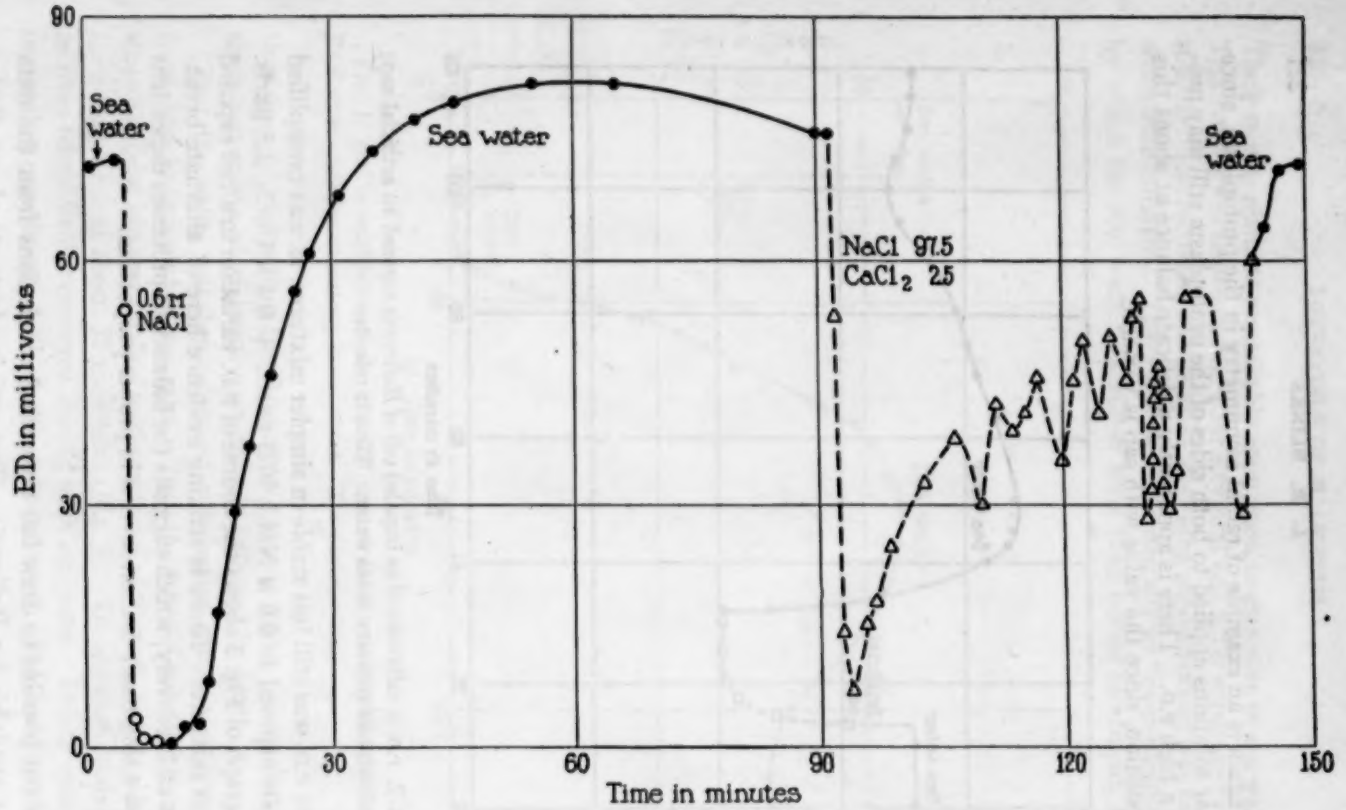

FIG. 3. P.D. in millivolts of an impaled cell of *Halicystis* exposed to (*a*) 0.6 M NaCl; (*b*) a balanced solution of 0.6 M NaCl 97.5 parts, and 0.4 M $CaCl_2$ 2.5 parts; with recovery in sea water. Time in minutes.

1. It shows a P.D. nearly ten times as large as does *V. macrophysa*, and four or five times as large as *V. ventricosa* (both being similarly impaled and immersed in sea water), and directed in the opposite sense. In these respects it is much more like *Nitella* in tap water both as to direction and magnitude of the P.D. The P.D. produced when sap is applied necessarily implies an asymmetric protoplasm.

2. Identification of the ions responsible for the E.M.F. appears difficult in view of the fact that a balanced solution is necessary for the production of any P. D. whatever. Systematic variation of the sea water composition is thus of doubtful value. Except for H^+ and SO_4^- there are no abundant ions of the sea water sufficiently different from those of the sap to give rise to an E.M.F. of 70 to 80 millivolts by concentration effect. That these two are probably not concerned was shown by changing their relative concentration in the sea water. Sulfate ion was doubled by the addition of Na_2SO_4 without effect on the P.D. The pH was changed from 8.2 to 6.0 without immediate effect. (Lower pH produced permanent alteration.)

Further study of these effects will be carried on. It is possible that the slow rise of P.D. observed after impalement is not due to a recovery but to an alteration such as a permanent lowering of E.M.F. at the outer or *X* layer. (This might be produced by the diffusion of salts into or out of the aqueous layer *W*.) Bridge measurements of intact cells show that they have a greater polarization response than the impaled cells, and we have increasing evidence of the expected correlation between polarizability and the bioelectric P.D. It is hoped that the study of *Halicystis* in conjunction with *Valonia* will assist in a general critique of the method of impalement.

SUMMARY

The cells of *Halicystis* impaled on capillaries reach a steady P.D. of 60 to 80 millivolts across the protoplasm from sap to sea water. The outer surface of the protoplasm is positive in the electrometer to the inner surface. The P.D. is reduced by contact with sap and balanced NaCl-$CaCl_2$ mixtures; it is abolished completely in solutions of NaCl, $CaCl_2$, KCl, $MgSO_4$, and $MgCl_2$. There is prompt recovery of P.D. in sea water after these exposures.

A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

I. The Reaction to Monochromatic Radiations

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(Accepted for publication, August 4, 1929)

During the past fifty years, many investigators have studied the bactericidal activity of ultra violet light, but a review of their reports shows that little has been learned about many essential factors in the reaction. There have been few contributions to a knowledge of the quantitative relations involved. Little precise information is available on differences in resistance of individual bacteria to monochromatic light and the consequent reaction curve of large numbers of organisms; on the effective range of ultra violet frequencies; on the relation between incident and absorbed energies at various wave lengths; on relations between time and intensity; on the temperature coefficient; on the action of polarized light; or indeed on any of the factors essential to the reaction on which an examination of its biophysical significance must be based.

But the ultimate object of the present study has not been simply to determine a set of coefficients for the bactericidal action of ultra violet light. This particular reaction was chosen for study because less individual variation is to be expected among bacteria than among higher forms of life. They are easily handled in quantity, and the death or survival of microorganisms provides a clearcut endpoint which may be observed and statistically recorded with unusual accuracy for biological material. They seemed to afford the best available opportunity for a quantitative study of an effect of certain frequencies in the ultra violet on protoplasm: with the prospect that the reaction of the bacterial cells might open leads for further studies on higher organisms.

It is obvious that in such work a considerable number of factors

must be controlled, and all but a selected one kept constant under standard conditions, while that one is varied experimentally. In the present study the measured variables were especially wave length, intensity, time, and lethal action, and, except when subject to special experiment, the factors kept as constant as possible were the character and age of the bacterial cultures employed and the composition, temperature, and hydrogen ion concentration of the medium on which the bacteria were exposed and subsequently allowed to multiply.

In quantitative studies energy of a single frequency, or a very narrow range of frequencies is essential: the selective absorption of radiant energy is characteristic of biological materials and a fundamental factor in their reaction to it. Since Grotthus it has been axiomatic that only energy that is absorbed can do work. The exposure of test objects to a whole gamut of radiation frequencies, even when the range is restricted by selective filters, introduces so many unknowns in partial absorption as to void any measurements of the effective energies. The importance of separating the radiations monochromatically has been recognized by Ward (1), Barnard and Morgan (2), Browning and Russ (3), Mashimo (4), and more recently by Bayne-Jones and von der Lingen (5), who exposed bacteria on a nutrient substrate in a quartz spectrograph, and so obtained spectral images of the result. But the equal importance of determining the incident energy required at each frequency to produce comparable effects has apparently been emphasized only by Hertel (6), who reported, however, on only six lines between 210 and 440 mμ. Bang (7) measured the periods of exposure (in seconds) necessary to kill at 20 mμ intervals (with the carbon arc), and tried to correlate the bactericidal effects with bolometric observations on his light source, and Coblentz and Fulton (8) have recently made careful radiometric observations on a few wide regions in the ultra violet defined by filters. But intensity measurements of monochromatic radiations in absolute units, and the correction of incident energies by the absorption coefficient of the objects under test apparently have not been undertaken. Yet it is obvious that physical and chemical analyses of the reaction, and quantitative comparisons with other biological ultra violet reactions can only be made on the basis of such information.

Methods

Light Source. Monochromatic ultra violet radiations were obtained from a vertical, quartz, air-cooled, high intensity mercury vapor arc, with tungsten anode, operating at 67 v., 5.5 amp. direct current. The mercury arc was chosen rather than a spark or a metallic open arc source, because of its steadiness and the intensity and separation of its principal spectral lines. While single frequencies or groups of frequencies within narrow limits are thus easily available, the ultra violet region between wave lengths 2253 and 3126 Å. u. is fairly well-covered by

the radiations at 2302, 2345, 2379, 2482, 2536, 2675, 2804, 2894, 2967, and 3022 Å. u.—all lines, or groups of lines, of sufficient intensity for practical use.

Quartz Monochromator. These specific ultra violet energies were separated and focused by means of a large quartz monochromator of special design.

The vertical quartz mercury lamp is placed on an optical bench, directly behind an adjustable entrance slit, curved to correct the spherical aberration of the quartz optical train. Radiations through the slit are rendered parallel by a crystal quartz planoconvex lens of 75 mm. aperture, and then pass through a Cornu quartz prism of 66 × 41 mm. face, and a large photographic shutter. A second 75 mm. crystal quartz lens then focuses the spectrum on a straight, vertical, adjustable exit slit, by which the desired frequency is isolated and passed to the surface of exposure. The bench supporting the shutter, collimating lens, and exit slit remain stationary, and different wave lengths are thrown upon the slit opening by rotation of the lamp housing, entrance slit, and collecting lens around the axis of the Cornu prism, maintained at minimum deviation by rotation through half the angle. Focus is obtained automatically by a synchronous adjustment of the lamp and entrance slit during their angular travel.

Energy Measurement. The intensity of the monochromatic radiations passed by the exit slit is measured, in the plane of the receiving surface, by thermopiles of special design, patterned after those described by Pfund (9), who kindly gave personal instruction in making them. These compensated, linear thermopiles are of 1.5 × 12 mm. surface, of about 5 seconds period, practically without creep at ordinary room temperatures, and, connected through a ballast resistance with a Leeds and Northrup high sensitivity galvanometer, give readings of about 1 cm. deflection at 2 meters scale distance for a flux of 1 erg mm.^{2} second (10^{-7} watt), a sensitivity ample for these energy measurements, as the protocols will show. At frequent intervals the thermopile in use was calibrated in absolute units against a Bureau of Standards carbon filament, incandescent lamp, (No. S 26), or a similar secondary standard.*

Test Objects. Although some measurements were made on a laboratory strain of *B. coli communis*, most of the observations here reported were made on a strain of *S. aureus*, originally obtained from Dr. C. G. Bull, and maintained on standard laboratory media for a period of years before these experiments were undertaken. This strain of cocci was chosen because of long adaptation to its environment, and because of the spherical shape of the single organisms, since orientation could not be controlled. During the experiments the strain was transplanted daily on beef infusion peptone 2 per cent agar, buffered at pH 7.4. This same medium was used as the substrate and covering layer in the tests.

For the quantitative determination of a bactericidal effect, it was essential, of

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* When energy measurements are given, it should be noted that the energy reported is the total per square millimeter of surface illuminated, and not the energy that falls upon each bacterium.

course, that no absorbing medium should intervene between the measured incident energy and the exposed bacteria. This, and the necessity that all organisms lie approximately in a plane, both during exposure and for subsequent colony counts, precluded the use of a fluid medium, and the following technique was finally adopted.

Experimental Procedure

Small Petri plates, 5.5 cm. in diameter, were attached with wax to 5.1 × 7.6 cm. glass microscope slides and partly filled with a layer of nutrient 2 per cent agar. A carefully made suspension of an 18 hour culture of *S. aureus*, with a transparency limit, or depth-of-disappearance of 30 cm. (10) was washed over the agar surface, the excess drained off, and the plate was allowed to stand in a vertical position until the excess fluid on the surface had evaporated. This method was found to give the most uniform distribution of organisms, so separated that subsequent colony counts could be readily made. The Petri plate was then covered with a crystal quartz plate (to compensate for the quartz window of the thermopile) and the glass slide was set vertically in a mechanical stage, adapted for the purpose, which was rigged on the monochromator so that the agar surface and the thermo-elements could be brought into position alternately, in the same plane, behind the exit slit. The five areas, 4 × 24 mm., to be exposed on each plate, separated by 4 mm. control areas, were then successively located and centered by readings on the mechanical stage. Timed exposures, with monochromatic radiation, and at measured intensities, were made at room temperature, between 20° and 22°C. Tests showed no appreciable variations within this temperature range.

After exposure, the bacteria-strewn surface of the Petri plates was covered with a second layer of nutrient agar at 39°C. In the earlier experiments the use of this covering layer of agar had not been developed, and it was necessary to incubate the plates at 37.5°C. under frequent inspection and to count the colonies in the exposed and control areas as soon as they became clearly visible and before confluent growth occurred. Then it was found that a second layer of agar, flowed on after exposure to cover the bacteria, dislodged an inconsiderable number of them (tens, out of the many thousands on the plate) and that no significant error was introduced by its use. On the contrary, the fixation of the bacteria between two layers of agar practically prevented confluence, and made possible an overnight incubation and a more accurate colony count.

For counting colonies, the exposed and control areas were located under the microscope by corresponding readings on a mechanical stage similar to that on the monochromator. A small central section of each area, measured between parallel lines and between stops on the mechanical stage, and corresponding approximately to the area of the thermopile junctions, was covered in each count.

The experimental errors which this method of estimating bactericidal action involves are obvious. Variations in the house current affect the light source.

Incident energy may not be uniform over the entire surface exposed. Bacteria may occasionally overlie and partly protect one another, and uneven distribution makes appreciable variations in the counts used as controls. This last source of error is the most conspicuous and is largely responsible for the common variations in the results of single exposures in parallel experiments, as shown in Chart 2. If single experiments were used at each wave length, irregular bactericidal curves would result. But each of the curves reported is the average of smoothed curves from a number of parallel experiments, and since errors due to irregular distribution fall indifferently above or below the line, the averages of these smoothed curves approximate closely the true course of the reaction. On the other hand, the use of such smoothed curves (Chart 1) to obtain the average of a number of experiments at each wave length precludes the inclusion of points on the final curves, lest they be interpreted as points of observation, rather than of statistical summary, and thus give a false impression of experimental accuracy. Therefore it will be noted that a number of such curves in this series are reported without points. The alternate method of presentation, when many observations in parallel experiments are scattered irregularly along a common energy gradient, is to collect the points into groups and average them at successive energy levels. For comparison this has been done in Table II, and the results plotted in Chart 2, together with all the single observations and with the curve obtained as the average of smoothed curves from each experiment. The close coincidence of the results of the two methods is apparent and would appear to justify the use of smoothed curves as the more acceptable representation of a continuously progressing reaction.

Before proceeding to an analysis of the experimental results, it may be stated that the effects reported are due to direct action of ultra violet light on the exposed bacteria. Browning and Russ (3), and Coblentz and Fulton (8) reported, and experiments in this series have confirmed the observation that exposure of an uninoculated agar surface to bactericidal wave lengths and intensities of ultra violet light has no measurable effect on the growth of microorganisms subsequently spread on the exposed areas, as compared with adjacent, unexposed controls.

In reports on the bactericidal action of ultra violet light, it is usual to consider first the range of frequencies which are effective. But, as will be shown later, this element in the problem is so intimately related to the specific absorption of energy at different wave lengths that it seems best to defer a consideration of the energies involved at different wave lengths until the typical reaction of bacteria at a single wave length has been examined.

The Reaction to Monochromatic Ultra Violet Light

The first experiments, then, deal with the typical reaction to monochromatic ultra violet light of an 18 hour culture of *S. aureus*, spread on nutrient agar plates, at a temperature of 20°C. and a hydrogen ion concentration of 7.4. The protocol of a single experiment at wave length 266 mμ is given in Table I and the smoothed curve of the findings in Chart 1.

TABLE I

A Single Experiment to Illustrate the Bactericidal Action of Monochromatic Ultra Violet Energy

S. aureus λ266 mμ 7/12/23
Galv. defl. 9.3 cm. Factor: 1 cm. defl. = 1.18 ergs per mm.² sec.
Incident energy = 11 ergs per mm.² sec.

Exp. sec.	2	4	6	8	10	12	16	20	24	30	Controls*	
Energy ergs	22	44	66	88	110	132	176	220	264	330	Plate	
Plate No.	1					2					1	2
Colonies	52	42	38	28	23	20	13	10	2	0	54–67	54–58
Per cent killed	15	31	38	54	62	64	77	82	96	100+	61	56
Plate No.	3					4					3	4
Colonies	70	64	56	43	39	32	19	11	7	0	87–80	78–82
Per cent killed	17	24	32	49	54	60	76	86	91	100+	84	80
Average killed per cent	16	28	35	52	58	62	77	84	94	100+		

* In later experiments control counts were made between each two adjacent exposed areas and averaged in pairs across the plate.

The ends of the curve would have to be obtained by extrapolation, for the least energy used killed some bacteria, and the greatest may have been more than sufficient to kill them all. The general trend of the reaction is to be seen, however, and the curve suggests that for the most part the relation of the incident ultra violet energy to its bactericidal action is logarithmic.

A more complete and accurate curve is obtained by averaging smoothed curves from a number of experiments at the same wave

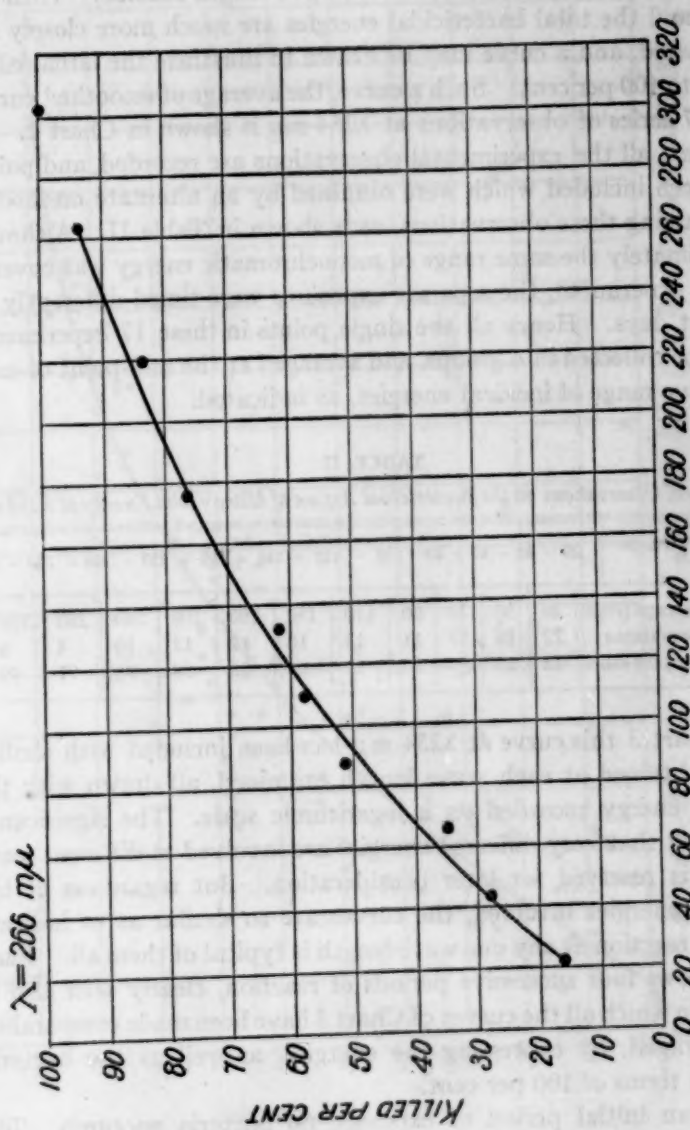

CHART 1. Course of the bactericidal action of monochromatic ultra violet energy in a single experiment.

length, which has been done at each wave length studied. Then the initial and the total bactericidal energies are much more closely approximated, and a curve may be drawn to illustrate the lethal effect from 0 to 100 per cent. Such a curve, the average of smoothed curves from 17 series of observations at λ254 mμ is shown in Chart 2. In this chart all the experimental observations are recorded, and points have been included which were obtained by an alternate method of summarizing these observations, as is shown in Table II. Although approximately the same range of monochromatic energy was covered in each experiment, the separate exposures were timed differently on different days. Hence all the single points in these 17 experiments had to be collected into groups, and averaged at the mid-point of each successive range of incident energies, as indicated.

TABLE II

Summary of Observations on the Bactericidal Action of Ultra Violet Energy at λ254 mμ

Energy levels (ergs per mm.²)	20 – 40	40 – 60	60 – 80	80 – 100	100 – 120	120 – 140	140 – 180	180 – 220	220 – 260	260 – 300	300 – 340
Point of average (ergs)	30	50	70	90	110	130	160	200	240	280	320
No. of observations...	22	18	17	11	13	10	17	12	10	4	6
Average per cent killed	13	25	36	50	59	66	76	88	94	97	99

In Chart 3 this curve at λ254 mμ has been included with similar curves obtained at each wave length examined, all drawn with the incident energy recorded on a logarithmic scale. The significance of the fact that very different energies are involved at different wave lengths is reserved for later consideration. But regardless of the absolute energies involved, the curves are so similar as to indicate that the reaction at any one wave length is typical of them all. Each curve shows four successive periods of reaction, clearly seen also in Chart 4 in which all the curves of Chart 3 have been made comparable, and averaged, by expressing the energies, as well as the bacteria killed, in terms of 100 per cent.

1. In an initial period of exposure no bacteria succumb. The energy incident before any bactericidal effect is observed is between 6 and 7 per cent of that required to kill all the organisms.

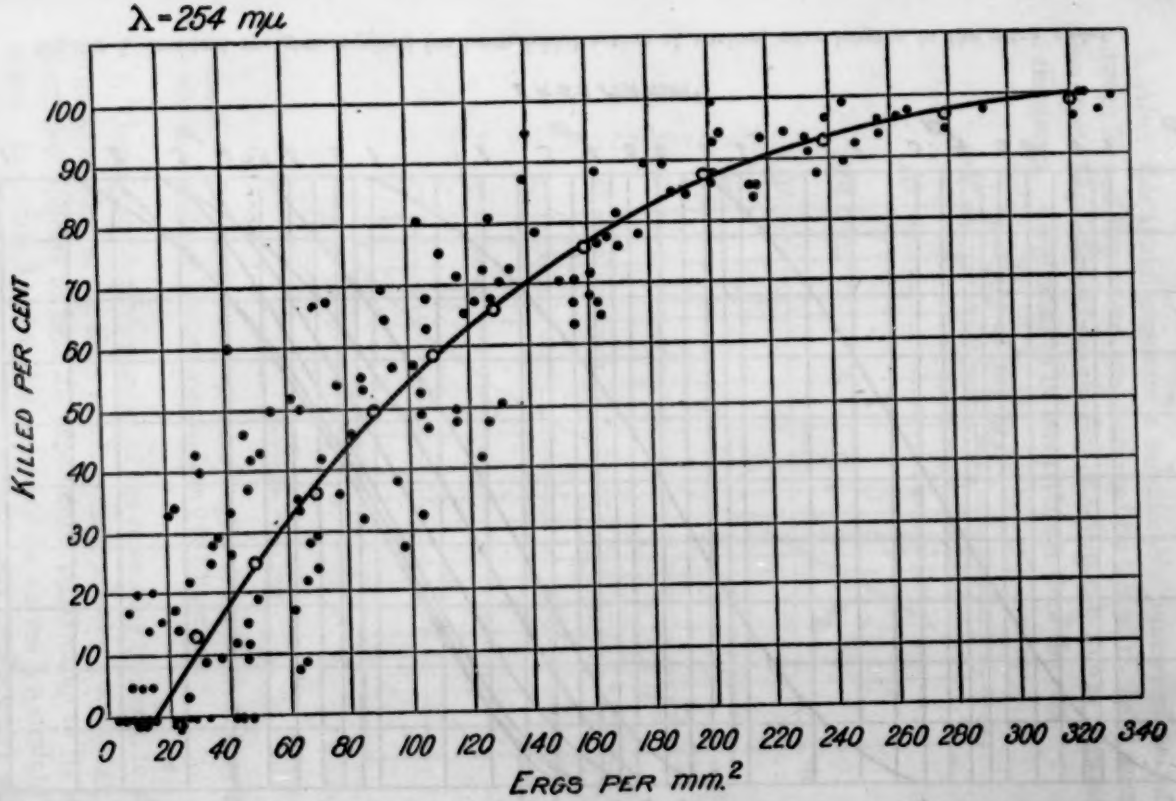

CHART 2. Smoothed curve of bactericidal action from a series of experiments. Points indicate single observations. Circles from Table 2.

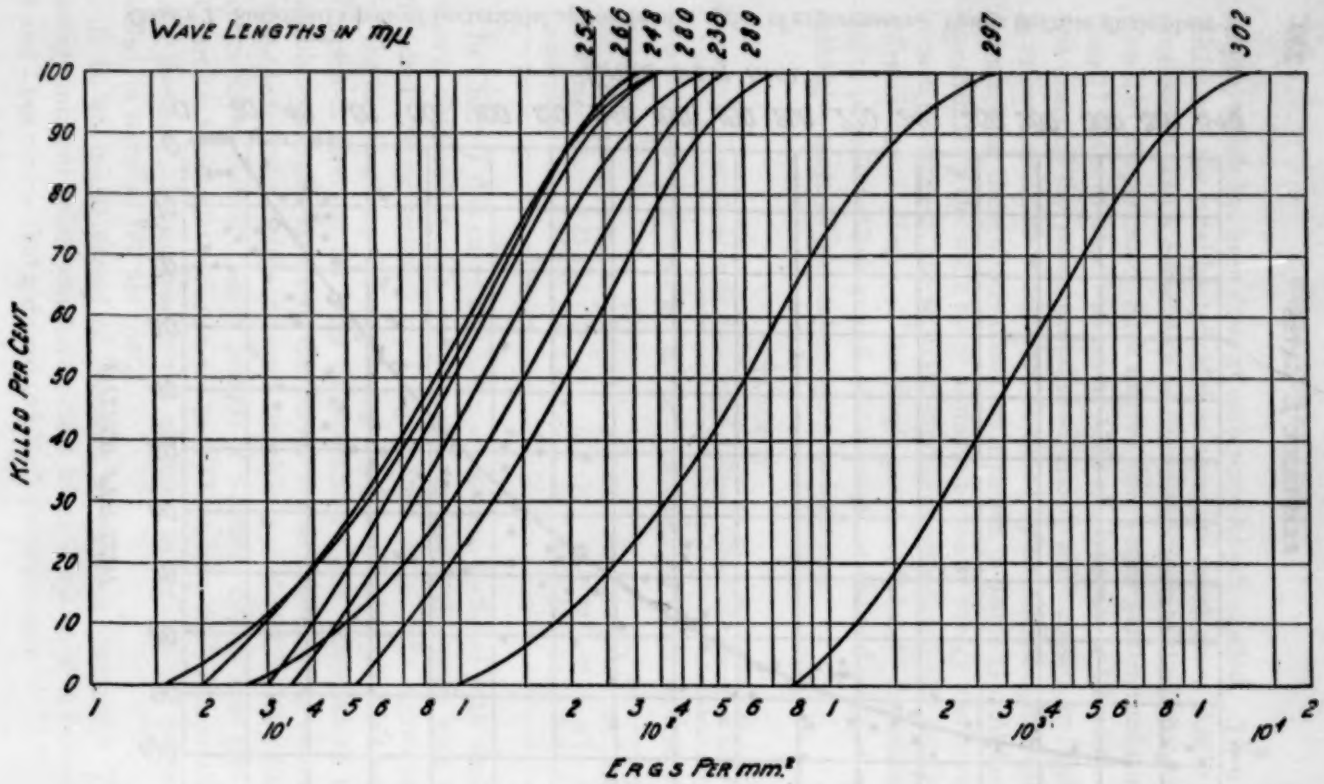

CHART 3. Incident energies required for bactericidal action at various wave lengths in the ultra violet.

2. After this initial exposure a considerable number of bacteria, between 20 and 30 per cent, are destroyed by less ultra violet energy than would be predicted from the rate of destruction for the remainder of the group. They seem to be less resistant than the rest.

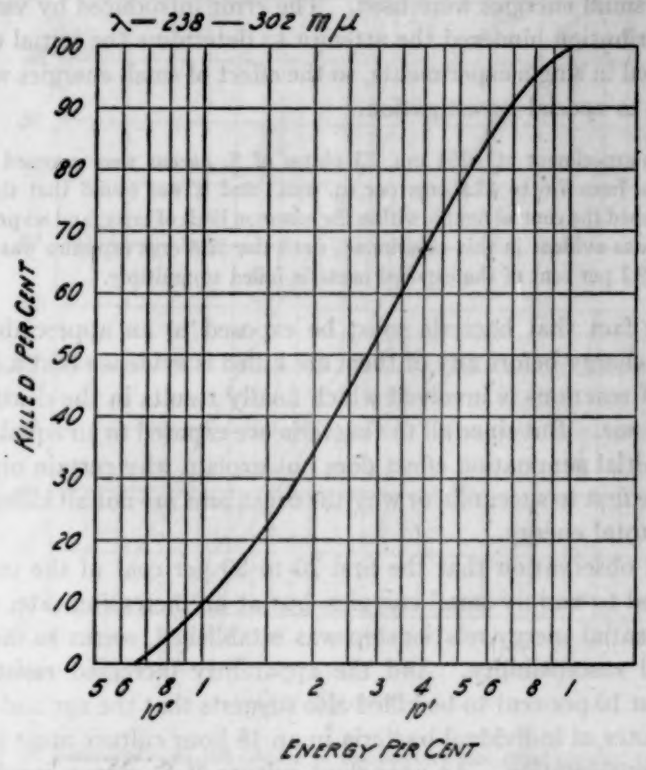

CHART 4. Smooth curve for all wave lengths shown in Chart 3, averaged by figuring the different incident energies from 0 to 100 per cent.

3. Then a considerable number of the remaining bacteria, to about 70 or 80 per cent of the total number, succumb along an energy gradient that appears to bear an exponential relationship to its lethal effect. In Charts 3 and 4, this section of the curves is a straight line.

4. In the final period a number of organisms remain which require an excess of energy to kill them.

Before the significance of the curve, as a whole, can be discussed,

consideration must be given to a number of factors that obviously affect it.

The presence of an initial period of exposure from the effects of which no bacteria succumb was attested by every experiment in which small energies were used. The error introduced by variations in distribution hindered the attempt to determine the initial energies involved in single experiments, so the effect of small energies was subjected to special investigation.

In an experiment at λ254 mμ, 23 plates of *S. aureus* were exposed to small energies, from 7.6 to 32.8 ergs per sq. mm., and it was found that the counts approached the control figures within the common limit of error, and no progressive action was evident in this experiment, until the 32.8 ergs exposure was reached. Then 18.2 per cent of the exposed bacteria failed to multiply.

The fact that bacteria must be exposed to an appreciable ultra violet energy before any of them are killed is evidence that a summation of reactions is involved which finally results in the death of the organisms. But since all the bacteria are exposed to an equal energy, this initial summation effect does not explain why certain organisms are the first to succumb, or why the organisms are not all killed by the same total energy.

The observation that the first 20 to 30 per cent of the cocci succumbed to unduly small energies, but at an increasing rate, until an exponential energy relationship was established, seems to indicate a special susceptibility. And the apparently increased resistance of the last 10 per cent to be killed also suggests that the age and relative resistance of individual bacteria in an 18 hour culture must be taken into consideration. An agar slant culture of *S. aureus*, incubated at 37.5°C., for 18 hours, has not yet reached the limit of multiplication, and contains many organisms but recently divided and in an active metabolic state. It is known that young bacteria are less resistant to ultra violet light than are older, resting organisms (11), and the following experiment shows the greater susceptibility of young individuals in the strain of *S. aureus* under test.

Plates were seeded with distilled water suspensions of broth cultures of *S. aureus*, grown at 37.5°C. for 4, 28, and 52 hours respectively, and were exposed to λ254 mμ. No differences could be detected in the

morphology of colonies which subsequently grew out in the various exposed and control areas, but Chart 5 (from smoothed curves) shows that the recently divided and genetically and metabolically active

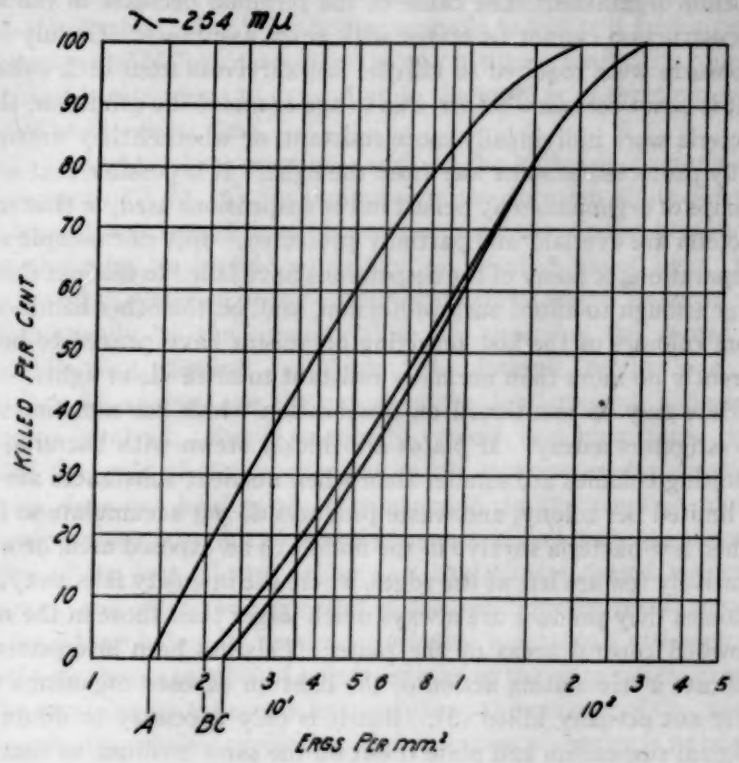

CHART 5. Differences in the course of the bactericidal reaction due to differences in age of the cultures employed.
A = 4 hrs. old.
B = 28 " "
C = 52 " "

bacteria in the 4 hour cultures were appreciably less resistant to the ultra violet energy. This relation between genetic activity and susceptibility to ultra violet radiation will be discussed in the final paper of this study.

It is interesting to note that the incident energy involved in the destruction of the 4 hour culture, in which most of the organisms would

fall in the same genetically active age group, apparently increases logarithmically from the beginning. This is additional evidence that the initial deaths in older cultures are of young and especially susceptible organisms. The cause of the terminal decrease in the rate of destruction cannot be stated with equal assurance. Unduly long exposures were required to kill the last survivors from each culture. But it is not certain whether, due to age or metabolic condition, these bacteria were individually more resistant, or whether they were partially protected in some way from the light. It is possible that small clumps of organisms may persist in the suspensions used, so that some bacteria are overlaid and partially protected. But microscopic slide preparations of many of the suspensions have failed to disclose clumps large enough to afford such protection, and, on the other hand, cocci from colonies of the last surviving organisms have proved to be inherently no more than normally resistant to ultra violet light.

Here may be mentioned an observation which has sometimes led investigators astray. If plates are thickly stewn with bacteria, the resulting colonies are smaller than when nutrient substances are not so limited per colony, and waste products do not accumulate so fast. When few bacteria survive in the middle of an exposed area, or when relatively few are left at the edges, where the intensity falls away, the colonies they produce are always much larger than those in the more crowded control areas of the plate. This has been interpreted to indicate a stimulating action of the light on exposed organisms that were not actually killed (3). But it is only necessary to dilute the original suspension and plate it out on the same medium, so that unexposed organisms are spaced as widely as the survivors in exposed areas are, to determine the source of the more active multiplication and consequent large colonies. Coblentz and Fulton (8) have noted the large colonies from surviving bacteria and have given this explanation of their development.

These experiments, therefore, give no evidence of a stimulating effect of ultra violet light in sublethal doses on the subsequent multiplication of bacteria. They show that there must be a summation of reactions due to radiant energy before any organisms succumb, and that the bacteria of an 18 hour culture show individual variations in their resistance to monochromatic ultra violet light.

DISCUSSION

The characteristic shape of the empirical curve for the bactericidal action of ultra violet light on *S. aureus* (Chart 4) invites an inquiry as to its significance. For it is another example to add to a growing list of experimental biological reactions in which, during most of its course, the reaction rate seems to depend upon the number of reacting units present at the given time.

It is typical of these reactions, such as the killing of bacteria by disinfectants (12, 13, 14), the limitation of the duration of life of fruit flies by untoward conditions of environment (15), or the hemolysis of erythrocytes by specific antibodies, or by ultra violet light (16) that the middle portion of the experimental curve may be reproduced mathematically by the equation that also describes the course of monomolecular chemical reactions. The formula tempts one to speculate, by analogy, on the nature of the fundamental biological reactions involved. But it is equally typical, and important, that toward the ends of the reaction curve the monomolecular reaction formula does not hold good. For example, in the reaction under discussion, there is an initial period of exposure, and consequent summation of its first effects, before any bactericidal action becomes apparent, and after the beginning of the reaction the reaction rate lags for a time before its maximum velocity is attained, although at first the maximum number of cells is exposed to the ultra violet energy. As the surviving units become relatively few, the velocity of the reaction again drops below prediction, and an excess of energy is required before the final bacteria succumb. The characteristic shape of these experimental curves for various biological reactions, with its similarity to the monomolecular reaction curve, and its equally essential differences, has given rise to extended discussions of its significance. It is generally recognized that differences in resistance must and do occur in heterogenous groups of biological units, such as fruit flies or bacteria, or erythrocytes, *in vitro*. If these differences in resistance are essential factors in the reaction of the individual, they must essentially modify the course of the reaction of the group. Under such circumstances, the course of the reaction depends on the distribution among the units of the factors causing resistance, and so the rate is

determined by probability rather than by the fundamental character of the reaction, and the appropriate curve with which to compare the rate is the "mortality curve" of insurance statistics (Chart 6). Then

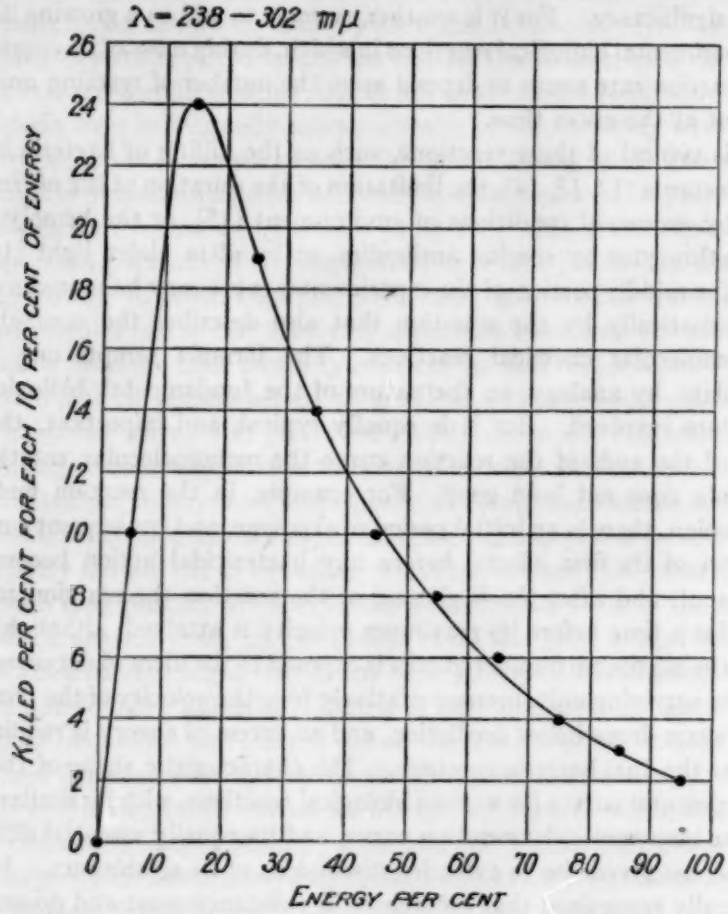

CHART 6. The data of Chart 4 refigured as a curve of probability.

the similarity of its middle portion to the course of a monomolecular reaction is to be ascribed to coincidence within the limits of experimental error—a distribution of resistance factors that simulates a logarithmic curve (15). On the other hand, proponents of the mono-

molecular reaction hypothesis insist that individual resistance varies only within relatively narrow limits, and when large numbers of units are under observation the differences in resistance may be disregarded, while the similarity to a monomolecular reaction persists.

These two opposed points of view are fully reviewed and defended by Brooks (16) and by Cohen (14). At best, the two interpretations affect only the theory of the course of the reactions and throw no light upon their fundamental nature in the various objects under test, as both writers are careful to insist.

The curves for the bactericidal effect of ultra violet light on *S. aureus* are evidently open to either interpretation. Experimental evidence of variations in individual resistance according to age stresses the importance of this factor in determining the course of the reaction. Partial elimination of the age factor by the use of young bacteria produces a reaction curve more nearly logarithmic at the start. But the most that can be said is that the rate of the fundamental reaction, whatever it may be, is undoubtedly modified by variations in individual resistance, and by variations in experimental conditions that mask its true course and make futile any attempt at exact interpretation. And the particular biophysical reactions in each bacterium that result in its death cannot be further analyzed merely from a series of observations on the incident energies that are involved at single wave lengths. A second essential factor in such an analysis—the relation of the incident energy at each wave length to that absorbed by the exposed bacteria—will be considered in a later paper of the series.

SUMMARY

In this first paper of a series on the bactericidal action of ultra violet light the methods of isolating and measuring monochromatic radiations, of preparing and exposing the bacteria, and of estimating the effects of exposure, are given in detail.

At all the different wave lengths studied the reactions of *S. aureus* followed similar curves, but occurred, at each wave length, at a different energy level. The general similarity of these curves to those for monomolecular reactions provokes a discussion of their signifiance, and emphasis is laid upon variations in susceptibility of individ-

ual organisms, due especially to age and metabolic activity, so that the typical curve seems to be best interpreted as one of probability.

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A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

II. The Effect of Various Environmental Factors and Conditions

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The first paper of this series (1) dealt with the reaction of an 18 hour culture of *Staphylococcus aureus* to monochromatic ultra violet energy, and it was shown that the course of the reaction was the same at each wave length studied. A consideration of certain factors, such as age and metabolic activity, in the resistance of individual bacteria gave a partial explanation of the course of the reaction among large numbers of organisms. But very different total incident energies were required at different wave lengths to produce these similar effects and an examination of energy relationships and the spectral limits of the bactericidal region was reserved for later consideration.

Before taking up the relation between incident energy and the coefficient of light absorption at different wave lengths, a relation essential to an analysis of the structural elements in bacteria which are affected by light, and to the nature of the resulting reactions, it seems desirable to estimate the effect on the reaction of various conditions of experiment and certain factors in the environment for which due allowance must be made.

The present paper, therefore, will deal with:

(1) The relation between the intensity of the incident energy and the time required for bacterial destruction (the Bunsen-Roscoe Law). (2) The spectral limits of bactericidal action. (3) The temperature coefficient of the bactericidal reaction. (4) The effect of the hydrogen ion concentration of the substrate. (5) The effect of polarization of the ultra violet radiation.

The Bunsen-Roscoe Law

Within the bactericidal zone examined in these experiments (λ238 to λ302 mμ) widely different incident energies were required at different wave lengths to produce similar effects, and since the available source intensities at these wave lengths differed considerably among themselves, it became necessary to know the effect of different intensities on total energies; *i.e.* to determine the validity for these experiments of the Bunsen-Roscoe reciprocity law of photochemistry (2) that when the product of intensity and exposure-time is constant a constant photochemical reaction results. The law does not hold with exactness in certain reactions, and has been modified by Schwartzschild (3) for photographic blackening. Coblentz and Fulton (4) studying the bactericidal action of ultra violet light and employing source intensities in the ratio of $\frac{1}{1} : \frac{1}{10} : \frac{1}{50}$ found that with low intensities a "proportionate increase in the time of exposure falls short of bringing about an equal killing effect." An intensity reduction to $\frac{1}{50}$ required an increase of × 75 in the exposure time to obtain a comparable reaction. This corresponds to a Schwartzschild exponent for the bactericidal reaction of 1.25. When such low intensities and correspondingly long exposures are used with living test objects like bacteria, the fact that the organisms may undergo metabolic or genetic changes during the exposure period must be taken into account, for such changes might themselves modify the reaction. Such wide differences in intensity as 1 and 50 did not enter into the present study, and Chart 1 illustrates the difference in effect of the extremes of intensity involved.

In the irradiation of *S. aureus at* λ266 mμ six series of plates were exposed to an average intensity of 21.6 ergs per mm.^{2} sec. and four to an intensity of 5.6 ergs per mm.^{2} sec. for proportionately longer periods. The averages of these determinations from smoothed curves of each series of bacterial counts (Chart 1*) show that at the lower incident intensity fewer bacteria were killed during most of the reaction period. But it is also evident that the curves for low and high incident intensity approach each other as the reaction progresses and the total energies involved in complete destruction are the same.

* The absence of points on the curves is explained in the first paper.

The differences in the curves would indicate, as suggested above, that it is the younger, metabolically or genetically active organisms which show the greater differences in response to differences in intensity. Since this difference in response varies continuously during the

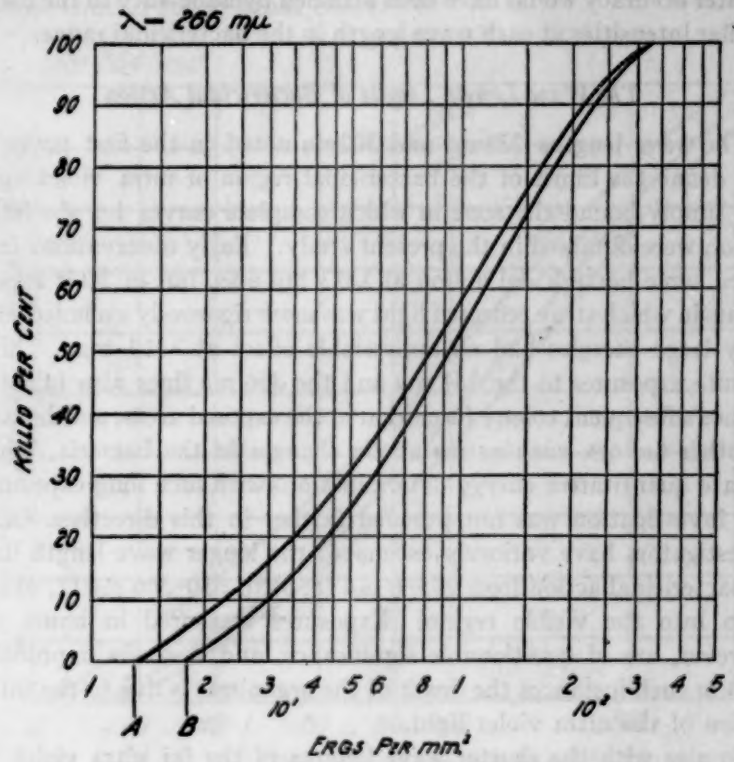

CHART 1. Effect on the bactericidal reaction of different intensities of incident radiation.

A = 21.6 ergs per mm.^{2} sec.
B = 5.6 " " " "

course of the reaction, decreasing as more and more organisms are killed, it is not possible to determine a Schwartzschild exponent except for one point on the curve. Thus at 10 per cent destruction the Schwartzschild modification ($I^q\ T = K$) of the Bunsen-Roscoe equation requires as an exponent, $q = 1.12$ while for 50 per cent destruc-

tion differences in intensity must be raised to the 1.08 power in order to determine a time factor that will give the same effect. Except for λ302 mμ the intensities did not differ at the various wave lengths as widely as those illustrated in this experiment, so it is doubtful if greater accuracy would have been attained by adherence to the use of similar intensities at each wave length in the bactericidal range.

The Wave Length Limits of Bactericidal Action

The wave lengths 238mμ and 302mμ noted in the first paper do not define the limits of the bactericidal region of ultra violet light, but simply bound the zone in which complete curves for the lethal action were obtained in the present study. Early observations indicated some bactericidal action at λ313 mμ also, but in later experiments in which stray reflected light was more rigorously excluded even very large energies had no appreciable effect at λ313 mμ. Thirty minute exposures to the 334 mμ and the 366 mμ lines also failed to reduce subsequent colony formation in the exposed areas, and because of other factors, such as metabolic changes in the bacteria, which vitiate quantitative energy determinations with such long exposures, the investigation was not pursued farther in this direction. Other investigators have variously estimated the longer wave length limit of bactericidal action from 295–6 mμ (5, 6) to 350–366 mμ (7, 4) and even into the visible region. Exposures measured in hours (8), however, are of questionable significance, and it seems improbable that in such instances the death of the organisms is due to the direct action of the ultra violet light.

So also with the shorter wave lengths of the far ultra violet, no attempt was made to find and measure a limit to the bactericidal zone. A few experiments with the weak mercury arc lines at λ234, 230, and 225 mμ established only the middle portion of the curves of lethal action, for the low intensities available at these wave lengths prolonged exposures and so increased the difficulty of obtaining accurate results.

Mashimo (5) using an iron spark before a spectrograph in which bacteria were exposed on nutrient agar plates found that with long exposures (150–300 minutes) the limits of bactericidal action on *B. coli* were λ2948–86 and λ1856 Å. u., the limit of air transmission. Lyman (9) had already shown a bactericidal effect of radiations below λ186

mμ and Bovie (10) had extended the lower limit to λ125 mμ by the use of a fluorite window before a hydrogen discharge tube. Although no energy measurements were undertaken he found these Schumann waves highly destructive to protoplasm, so that only short exposures were required for a lethal effect.

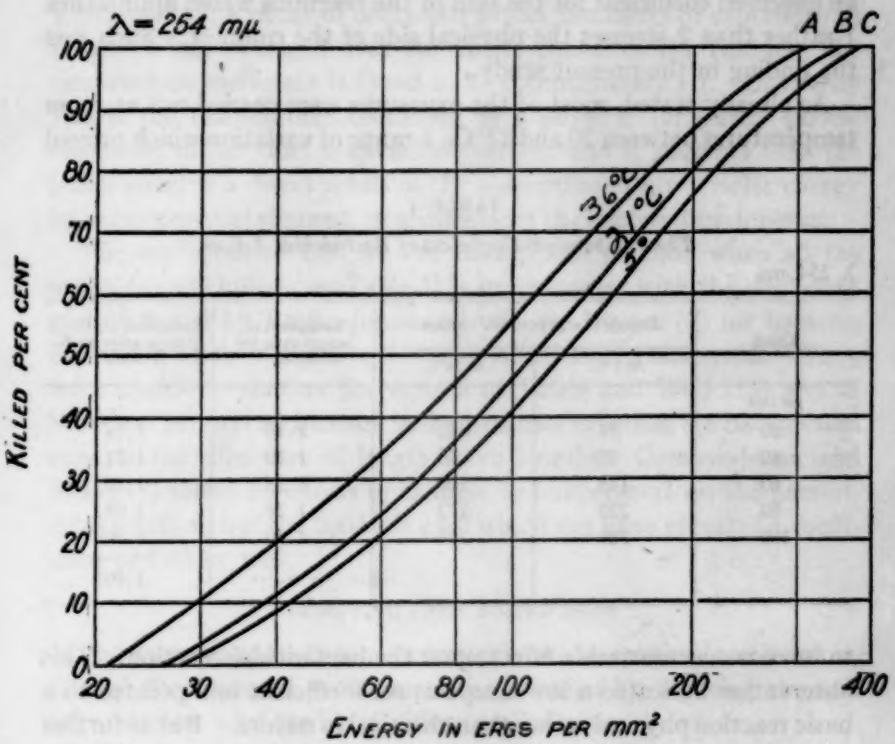

CHART 2. Temperature coefficient of bactericidal action.
A at 36°C.
B at 21°C.
C at 5°C.

The Temperature Coefficient of the Bactericidal Reaction

Of primary importance in determining whether the bactericidal activity of ultra violet light is physical or chemical in nature is the temperature coefficient of the reaction. For although, as Cohen (11) points out, the generalization that the temperature coefficient of physi-

cal reactions is about 1 and of chemical reactions usually above 2 rests on a purely empirical basis, enough experimental evidence has accumulated to warrant a deduction when the observations are clearcut and striking. Even when, in biological experiments, reactions with different temperature coefficients may be progressing simultaneously (12), an observed coefficient for the sum of the reactions which approaches 1 rather than 2 stresses the physical side of the complex. Such was the finding in the present study.

As already stated, most of the exposures were carried out at room temperatures between 20 and 22°C., a range of variation which proved

TABLE I

The Temperature Coefficient of Bactericidal Action

λ 254 mμ

Killed	Energy in ergs per mm.² required		Reciprocal of energy ratio for 31°C.	Reciprocal for 10°C. (temperature coefficient)
	At 5°C.	At 36°C.		
per cent				
20	58	40	1.45	1.13
40	98	70	1.40	1.11
60	148	110	1.35	1.10
80	220	172	1.28	1.08
100	380	316	1.20	1.06
				1.096

to have no demonstrable effect upon the bactericidal reaction. This observation indicated a low temperature coefficient and pointed to a basic reaction physical rather than chemical in nature. But as further evidence, a special series of experiments was made over a much wider temperature range in order to obtain the coefficient for a rise of ten degrees in the environment.

Three series of observations at λ254 mμ were run in parallel, with the agar medium on which the *S. aureus* were strewn maintained at 5°, 21°, or 36°C. during exposure. Thus three groups of bacteria were exposed to the same range of ultra violet energy, but underwent at different temperatures the reactions that resulted in their deaths. The smoothed curves summarizing the experiments are shown in Chart 2, and from these curves the temperature coefficient of the

bactericidal reaction may be figured, for the coefficient would be the reciprocal of the ratio of energies required to produce the same effects at a difference of 10° in the reaction temperature. The ratios of energies involved at 5° and at 36° are shown in Table I, and the reciprocal of these ratios for a change of 10° is found to vary between 1.06 and 1.13, a range of difference within the limits of experimental error. When averaged over the entire course of the reaction, the temperature coefficient is found to be approximately 1.1. Obviously this is the temperature coefficient of a physical (or purely photochemical) rather than a chemical reaction and it suggests that the lethal effect is a direct result of the absorption of ultra violet energy by some essential element, or elements, of the bacterial protoplasm.

The coefficient of 1.06 for the bactericidal reaction when all the organisms are killed (see Table I) is in agreement with the coefficient of 1.05 found by Bayne-Jones and von der Lingen (7) for bacteria exposed in a fluid medium to the total radiations of a zinc spark. They were unable to confirm the reports of Thiele and Wolf (13) and of Wiesner (14) that an increase in temperature extended the bactericidal zone in the direction of longer wave lengths. Cernovodeanu and Henri (8) found no effects of changes in temperature on the bactericidal reaction, but the methods used would not have revealed a coefficient of 1.05 or 1.06.

Hydrogen Ion Concentration

The nearest approach to a variation in the hydrogen ion concentration of the bacterial protoplasm was afforded by exposing the test organisms on agar media of different alkalinities.

The veal-peptone 2 per cent agar medium used uniformly in the series was made up without buffer, and flasks of it were titrated with $\frac{N}{2}$ HCl to pH 4.5 and 6.0, estimated colorimetrically, and with $\frac{N}{2}$ NaOH to pH 7.5, 9.0 and 10.0 respectively. Small Petri plates of these media were then washed with *S. aureus* and exposed in the usual manner to λ266 mμ of the mercury arc spectrum. After exposure all the plates were layered with buffered agar at pH 7.4 and incubated at 37.5°C. overnight. The buffered agar did not bring the hydrogen ion concentration of all the media to 7.4 but to values between 5.5 and 8.2, and within this range no appreciable difference was observed in the number of colonies in equal control areas.

Counts of 9 plates at each pH (in 3 parallel experiments) are averaged in Table II and the corresponding smoothed curves are shown

TABLE II

Bacteria Killed, Per Cent, at Different Hydrogen Ion Concentrations

λ 266 mμ

Energy in ergs per mm.²: pH	100	150	200	250	300
4.5	43	63	73	85	95
6.0	44	62	77	89	96
7.5	42	67	77	87	96
Average 4.5, 6, 7.5.....	43	64	76	87	96
9.0	53	71	83	93	99
10.0	52	69	80	89	97

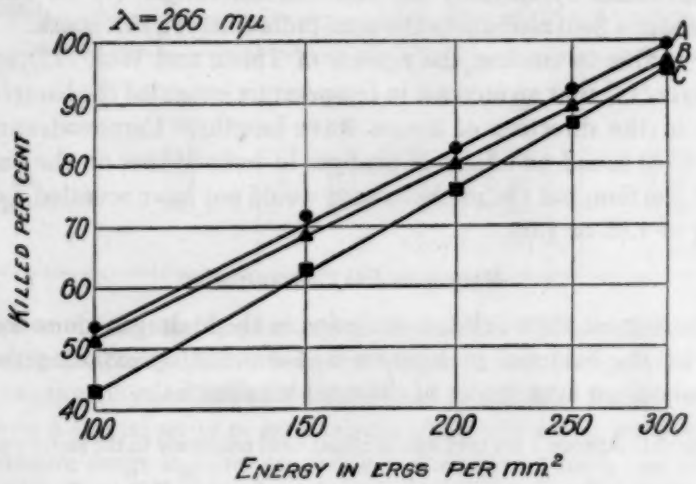

CHART 3. Bacteria killed per cent at various hydrogen ion concentrations of the medium.

A = pH 9.0 ●

B = pH 10.0 ▲

C = pH 4.5, 6.0, and 7.5 (averaged) ■

in Chart 3, except that the points for pH 4.5, 6.0 and 7.5 are so nearly alike that they have been averaged and a single curve drawn through

them in the chart. The figures for pH 9 and 10 approach this average within the accepted limit of error of the method, but since they are uniformly higher, they may indicate a real difference, at these hydrogen ion concentrations, in the susceptibility of *S. aureus* to ultra violet light. Possibly the higher death rates are due to a less favorable environment for the subsequent recovery and multiplication of damaged cells. It is evident that the difference is not so great as to warrant positive deductions to be drawn from these experiments, and one may conclude, on the contrary, that with the methods employed, variations in the hydrogen ion concentration of the substrate between 4.5 and 7.5 would have no appreciable effect upon the bactericidal reaction. Within the range pH 4.5 to 9.0 these results are in accord with those of Bayne-Jones and von der Lingen (7) who found but slight variation in the time required to kill staphylococcus in an alkaline fluid medium. When acid media were used, however, the bactericidal action was greatly accelerated and at pH 2 all the exposed bacteria were killed in 2 seconds exposure, although at pH 6 to 8 the same radiations had required 22 seconds for the same effect.

The Effect of Polarization of the Incident Light

Unless test objects are crystals, or have some plane of symmetry in which they may be placed with respect to a plane of polarization it is difficult to see how polarized light can have a special and significant effect upon them. Yet many examples could be collected from folk lore and from the literature of the alleged biological action of polarized, as distinguished from unpolarized light. This series of experiments afforded an opportunity to determine with measured energies under controlled conditions whether polarization made any difference whatever in the bactericidal action of ultra violet radiation.

After passage through the monochromatic illuminator, the line at 254 mμ was polarized by reflection from a plane quartz surface at the polarizing angle so that no measurable energy traversed a Nicol prism set at 90° to the plane. After removal of the Nicol prism from the path, this polarized monochromatic light was measured in ergs per mm.^{2} sec. by means of the thermocouple and galvanometer, and then used to irradiate *S. aureus* spread on agar plates in the manner already described. Controls were obtained by "depolarizing" the light by a polished plate of crystal quartz. Rotation of a Nicol prism in the beam of depolarized light no

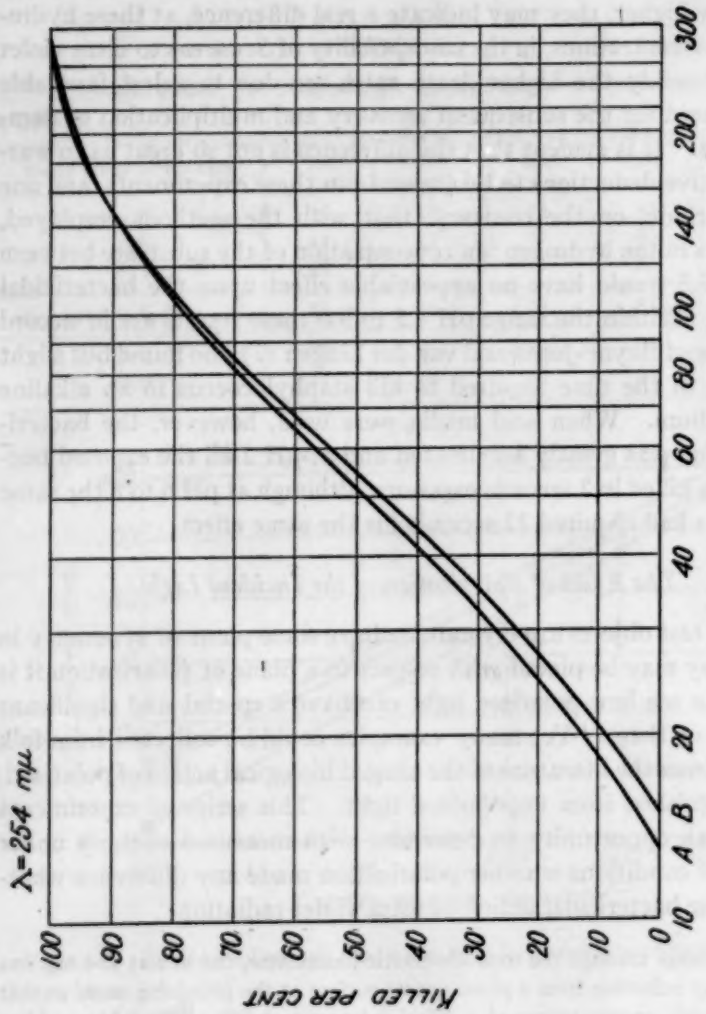

CHART 4. A comparison of the action of (*A*) depolarized and (*B*) polarized ultra violet energy.

longer varied its intensity appreciably. The energy incident on the agar plates was again measured with thermocouple and galvanometer so that a quantitative comparison between the bactericidal action of polarized and depolarized light could be made.

With this set-up eleven series of determinations were made, and the averages are shown in Chart 4. It is obvious that the effects of the polarized and depolarized light are identical, for the differences in the curves are well within the limit of error. That these experiments with polarized and depolarized energy are closely comparable with those done more than two years earlier with unpolarized light (Chart 2, Paper I) is further evidence that plane polarization has no observable effect upon the bactericidal activity of ultra violet radiation.

SUMMARY

1. Wide differences in the intensity of incident ultra violet energy are not accurately compensated by corresponding changes in the exposure time, so that the Bunsen-Roscoe reciprocity law does not hold, strictly, especially for bactericidal action on young, metabolically and genetically active bacteria. In the present series of experiments, however, the energies used at various wave lengths did not differ by so much as to cause a significant error in the reported reactions.

2. The longer wave length limit of a direct bactericidal action on *S. aureus* was found to be between 302 and 313 mμ. The shorter limit was not determined because the long exposures required vitiate quantitative results. Bactericidal action was observed at λ225 mμ.

3. The temperature coefficient of the bactericidal reaction approaches 1 and thus furnishes empirical evidence that the direct action of ultra violet light on bacteria is essentially physical or photochemical in character.

4. The hydrogen ion concentration of the environment has no appreciable effect upon the bactericidal reaction between the limits of pH 4.5 and 7.5. At pH 9 and 10 evidence of a slight but definite increase in bacterial susceptibility was noted, but this difference may have been due to a less favorable environment for subsequent recovery and multiplication of injured organisms.

5. Plane polarization of incident ultra violet radiation has no demonstrable effect upon its bactericidal action.

In a third paper of this group the ratios of incident to absorbed ultra violet energy at various wave lengths and the significance of these relations in an analysis of the bactericidal reaction will be discussed.

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THE KINETICS OF PENETRATION

I. Equations for the Entrance of Electrolytes

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Difficulties in picturing the mechanism of permeability, and in deciding whether ions can enter clearly indicate the need of mathematical treatment. The brief outline which follows represents merely a preliminary attempt in this direction.

A convenient method of approach is to consider the penetration[1] of a weak acid, HA, into a living cell when it is the only external solute present (conditions being such that penetration of molecules follows a curve of the first order[2]). If we assume that the activity coefficient is 1 we may define the permeability of the protoplasm as equal to the quantity of penetrating substance passing through 1 sq. cm. of protoplasmic surface into the vacuole in unit time under unit concentration (activity) difference.[3]

Case I. Molecules Alone Enter. Let us begin by writing

$$P_M = H_M D_M G_M$$

where P_M is the permeability of the protoplasm for molecules, D_M is the net rate of progress of molecules through the protoplasm,[4]

[1] *Cf.* Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 192.

[2] *E.g.*, when the inside is stirred by convection currents or by protoplasmic motion and the outside is also stirred. According to J. H. Northrop (private communication) the curve may in some cases be of the first order even if there is no internal stirring, but in that case the time required to diffuse from the surface to the interior of the cell may become the determining factor.

That the penetration may follow a curve of the first order when the sap is stirred by protoplasmic motion is evident from the experiments of Irwin (*J. Gen. Physiol.*, 1925–28, **8**, 147).

[3] This formula follows in a general way that of Northrop (*J. Gen. Physiol.*, 1928–29, **12**, 435) for penetration through collodion membranes. For molecules we have unit concentration difference when $M_o - M_i = 1$.

[4] This is equal to the reciprocal of the time required to pass through and depends

G_M is a collision factor (such that if one-half of the molecules reaching the surface pass through the protoplasm[5] we may put $G_M = 0.5$); and H_M is a proportionality factor. Let us assume for simplicity that a base ROH (produced by the cell) is the only solute of importance inside and that it tends to act as a buffer[6] and yields the

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not only on the thickness of the protoplasm but on other factors which may be chemical or physical. In the case of ions the value of D will be intermediate between that of the slower and that of the faster ion.

In case we are considering only penetration through the outer surface into the protoplasm (not into the vacuole) D_M and P_M must be modified accordingly.

If the diffusion gradient be kept constant a steady state will be reached in which the amount entering the outer surface of the protoplasm in unit time will be equal to the amount leaving the inner surface and passing into the vacuole (*cf.* Northrop, J. H., *J. Gen. Physiol.*, 1928–29, **12**, 435). We assume for convenience that the protoplasmic layer is so thin that we may neglect the time necessary to reach the steady state (but no actual steady state is assumed for the cases discussed in this paper since the diffusion gradient is not constant).

[5] The factor G is intended to cover the situation regardless of whether the penetration depends on passage through pores, on chemical combination, or on solubility (*cf.* Northrop, J. H., *J. Gen. Physiol.*, 1928–29, **12**, 435). The presence of pores in the surface of the protoplasm would seem less probable since the rounding up of drops of protoplasm in water indicates that the surface acts like a liquid but it is not impossible that a thin film (solid or gel) might overlie the liquid (as in the case of a drop of mercury covered with a solid film). In that case water-soluble substances might pass through the pores and lipoid-soluble substances through the substance surrounding the pores.

At any rate we must consider penetration through more than one phase since it would appear that protoplasm may contain several layers. (*Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 391; also Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, **11**, 193.)

[6] In this paper we shall neglect any change of internal pH produced by the entrance of small amounts of HA or NaA unless otherwise stated. As an example of what is meant let us assume that at the start $M_o = 10^{-4}$ mols per liter, $A_o = 10^{-8}$, $H_o = 10^{-8}$, $ROH = 10^{0.00432}$, $R = 10^{-3.99784}$, $OH_i = 10^{-3.99784}$, $H_i = 10^{-10.00216}$ and $M_i = A_i = 0$, the corresponding values at equilibrium being $M_{oe} = 10^{-4}$, $A_{oe} = 10^{-8}$, $H_{oe} = 10^{-8}$, $ROH_e = 10^{0.004319}$, $R_e = 10^{-3.99568}$, $OH_{ie} = 10^{-4}$, $H_{ie} = 10^{-10}$, $M_{ie} = 10^{-4}$ and $A_{ie} = 10^{-6}$. We assume that the dissociation constant K_B for $ROH = 10^{-8}$ and for $HA = 10^{-12} = K$. These figures satisfy the conditions for a Donnan equilibrium since $(H_{oe})\ (A_{oe}) = (H_{ie})\ (A_{ie})$: also $R_e + H_{ie} = A_{ie} + OH_{ie}$. It may be noted that there is little change in pH value due to the entrance of HA (*i.e.*, the change is from $H_{ie} = 10^{-10.00216}$ to $H_{ie} = 10^{-10}$).

cation R^+ which is unable to pass out. If the dissociation constant of HA is K we have $(H_o)(A_o) = KM_o$ and $(H_i)(A_i) = KM_i$, where the subscript o denotes outside and i inside, M denotes the concentration (activity[7]) of molecules, and H and A that of the ions. If we may assume that the presence of ROH provides the condition for a Donnan equilibrium we may consider that at equilibrium $(H_{oe})(A_{oe}) = (H_{ie})(A_{ie})$ and $M_{oe} = M_{ie}$, where the subscript e denotes concentration (activity[7]) at equilibrium.

We assume that the internal volume is 1 liter[8] and that the outside concentration is kept constant.[6] Hence at the beginning (when $M_i = 0$) if $M_o = 1000$ millimols per liter and $P_M = 0.01$ the amount entering in the first unit of time is $P_M M_o$ and if we assume for convenience that nothing passes out during the first unit of time the concentration at the end of the first unit of time is $P_M M_o$ or 10 millimols per liter. How much of this remains in molecular form after entering depends on the internal pH value which determines the fractional concentration, F_M, of molecules inside.[9] We may put $F_M = \frac{M_i}{M_i + A_i}$ and since $A_i = \frac{KM_i}{H_i}$ we have

$$F_M = \frac{M_i}{M_i + \frac{KM_i}{H_i}} = \frac{1}{1 + \frac{K}{H_i}}$$

The fractional concentration of A^- inside, F_A, is

$$F_A = \frac{A_i}{A_i + M_i} = \frac{A_i}{A_i + \frac{H_i A_i}{K}} = \frac{1}{1 + \frac{H_i}{K}}$$

We shall for convenience assume F_M and F_A to be constant for any one time curve.

[7] In case the activity coefficient does not equal 1 the treatment must be altered accordingly but this involves no difficulty.

[8] *I.e.*, the total internal volume of all the cells taken together is 1 liter.

[9] If the pH value inside falls during the penetration F_M will increase and the velocity constant V_M will become greater since $V_M = P_M F_M$; it is as though the temperature were to be slowly raised during a chemical reaction of the first order. (The same would be true if the pH value rose during the penetration of a base.)

It is evident that the inward diffusion is proportional to P_M and to the difference in concentration (*i.e.*, to $M_o - M_i$),[10] so that if $S_i = M_i + A_i$ the net amount entering in unit time may be regarded as

$$\frac{dS_i}{dt} = P_M(M_o - M_i)$$

It may be more convenient in the subsequent discussion to assume that there is an inward diffusion proceeding as though M_i and A_i were always zero and an outward diffusion proceeding as though M_o and A_o were always zero. In that case the amount entering in unit time would be $P_M M_o$ and the amount coming out would be $P_M M_i$, so that the net amount entering would be

$$\frac{dS_i}{dt} = P_M M_o - P_M M_i = P_M(M_o - M_i)$$

Since $M_o = M_{ie}$ we have

$$\frac{dS_i}{dt} = P_M(M_{ie} - M_i)$$

where the subscript e denotes concentration at equilibrium. If we multiply the right-hand side of the equation by $\frac{F_M}{F_M}$ we have

$$\frac{dS_i}{dt} = P_M F_M\left(\frac{M_{ie}}{F_M} - \frac{M_i}{F_M}\right) = P_M F_M(S_{ie} - S_i)$$

Putting $P_M F_M = V_M$ we have

$$\frac{dS_i}{dt} = V_M(S_{ie} - S_i)$$

and

$$V_M = \frac{1}{t} \ln_e \frac{S_{ie}}{S_{ie} - S_i}$$

We may also put

$$\frac{dA_i}{dt} = \frac{dS_i}{dt} F_A = V_M(F_A S_{ie} - F_A S_i) = V_M(A_{ie} - A_i)$$

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[10] If a substance must combine with a constituent of the protoplasm in order to enter, the rate of penetration is not a linear function of $M_o - M_i$ but may closely approximate it in many cases. This would also apply to the penetration of ions.

and

$$V_M = \frac{1}{t} \ln_e \frac{A_{ie}}{A_{ie} - A_i}$$

Also[11]

$$\frac{dM_i}{dt} = \frac{dS_i}{dt} F_M = V_M(M_{ie} - M_i)$$

and[12]

$$V_M = \frac{1}{t} \ln_e \frac{M_{ie}}{M_{ie} - M_i}$$

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[11] This may be illustrated as follows. If the time unit be sufficiently short we may regard the rate as uniform during any one interval and if at the beginning $M_i = A_i = 0$ we may assume that during the first interval of time the egress is negligible. If $M_o = 1000$ millimols per liter, $P_M = 0.01$, and $F_M = 0.1$ the amount leaving the external solution in the first unit of time is $P_M M_o = (0.01)(1000) = 10$ of which all but 1 is converted into ions after entering so that the increase in M_i is $P_M M_o F_M = (0.01)(1000)(0.1) = 1$. Putting $P_M F_M = V_M$, the apparent velocity constant of the process, we have

$$V_M = \frac{1}{t} \ln_e \left(\frac{M_o}{M_o - M_i} \right) = \ln_e \left(\frac{1000}{1000 - 1} \right) = 0.001$$

(whence $P_M = 0.01$). During the next unit of time a part of this escapes by outward diffusion: if we regarded this as $P_M M_i$ we should have $P_M M_i = (0.01)(1) = 0.01$, but when this has passed out we have inside 0.99 M_i and 9 A_i so that $M_i + A_i = 9.99$; but ions must combine until we have $M_i = F_M(M_i + A_i) = (0.1)(9.99) = 0.999$ so that the real loss of $M_i = 1 - 0.999 = 0.001$ or $= P_M M_i F_M = (0.01)(1)(0.1) = 0.001$. The amount of M_i coming in during the second unit of time is, as before, $P_M F_M M_o = 1$. The net increase in M_i in the second unit of time, $\frac{\Delta M_i}{dt}$, is the difference between the incoming and the outgoing quantities or $P_M M_o F_M - P_M M_i F_M = P_M F_M(M_o - M_i) = (0.01)(0.1)(1000 - 1) = 0.999$. Putting $P_M F_M = V_M$, the apparent velocity constant of the process, we have

$$\frac{\Delta M_i}{dt} = V_M(M_o - M_i)$$

and for the first two units of time we have

$$V_M = \frac{1}{t} \ln_e \frac{M_o}{M_o - M_i} = \frac{1}{2} \ln_e \frac{1000}{1000 - (1 + 0.999)} = 0.001$$

whence $P_M = 0.01$ (this value is approximate only; the smaller the assumed value of V_M the nearer will be the agreement of the calculated and assumed values).

[12] This equation assumes that the partition coefficient between the protoplasm and the external solution is 1: cases where it differs from 1 will be discussed later.

This may be checked by considering the condition at equilibrium where we know that the rate of increase of M_i or $\frac{dM_i}{dt} = P_M M_o F_M$ must be equal to the rate of decrease of M_i or $\frac{dM_i}{dt} = P_M M_i F_M$; hence we have $P_M M_o F_M = P_M M_i F_M$: this is correct because at equilibrium $M_o = M_i$.

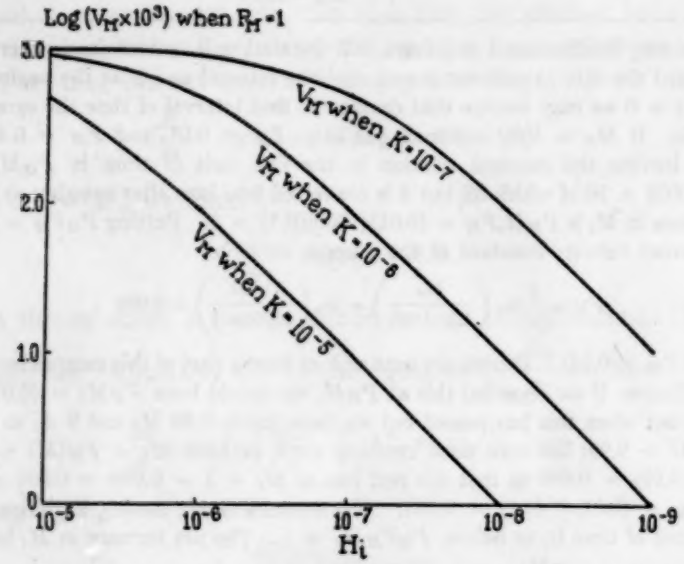

FIG. 1. Shows how V_M ($= P_M F_M$) varies when P_M is constant ($P_M = 1$) but K, H_i (and in consequence F_M) vary. K, H_i and F_M are taken as constant during any one time curve or process of penetration.

In Fig. 1 are shown the magnitudes of V_M for various values of K (the ionization constant of HA) and of internal pH (and hence of F_M) when $P_M = 1$, *i.e.* when $V_M = F_M$.

It should be noted that if M_o remains constant a decrease of F_M (caused by an increase in pH value inside) means an increase of S_{io} (the total internal concentration at equilibrium, as indicated in Fig. 2) as well as a decrease of V_M.

CORRECTION

On page 267, Vol. **13**, No. 2, November 20, 1929, the bottom line should read

$$\frac{dS_i}{dt} = P_M(M_o - M_i)$$

On page 275, the fifth line should read

where $P_M + P_A K = 1$ this becomes $\left(\frac{dS_i}{dt}\right)_b = M_o = M_{ie}$

In order to compare the permeability of the protoplasm for different acids (penetrating as molecules only) we may ascertain the values of V_M and F_M. The latter tends to rise as the acid penetrates,[13] but if the amount penetrating is small and the buffer action of the cell sufficiently great the change in F_M might be negligible. The error would be minimized by taking the rate at the very start (before much change was brought about by penetration), but here the experimental difficulties might make it necessary to construct a

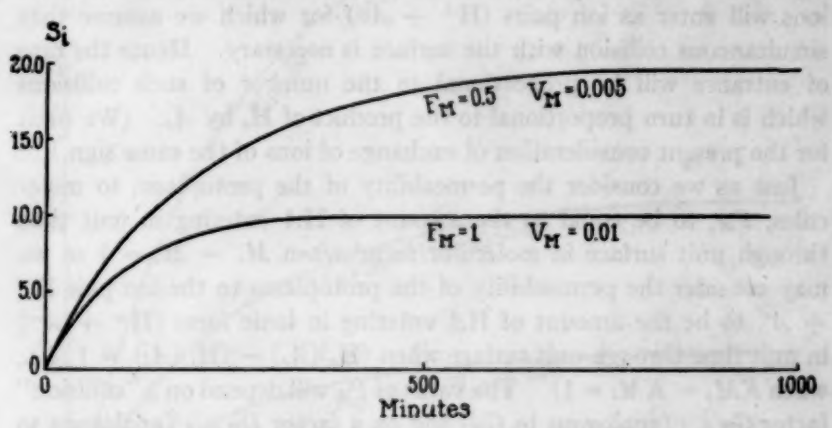

FIG. 2. Calculated time curves of S_i (total amount inside) when molecules alone penetrate and M_o and P_M are constant ($P_M = 1$) but F_M varies and in consequence V_M varies. It is assumed that $M_o = M_{i_o} = 10$ for both curves. The equation is $V_M = P_M F_M = \frac{1}{t} \ln_e \frac{S_{i_o}}{S_{i_o} - S_i}$.

The same curves would serve if ions alone entered by putting V_A in place of V_M and $P_A K = 1$ in place of $P_M = 1$ or if ions and molecules entered by putting V_{MA} in place of V_M and $P_M + P_A K = 1$ in place of $P_M = 1$. In all these cases the rate rises as the external pH value decreases.

portion of the time curve and extrapolate to zero time. At the start, when $M_i = 0$ and $A_i = 0$ we may neglect F_M since when $M_i = 0$ the equation

$$\frac{dS_i}{dt} = P_M F_M (M_o - M_i)$$

[13] This would also be true of the penetration of a weak base.

becomes

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o$$

where $\left(\frac{dS_i}{dt}\right)_b$ is the rate when $M_i = A_i = 0$.

Case II.—Let us now consider the penetration of ions.[14] We assume that the outer surface of the protoplasm consists of a non-aqueous layer through which A^- cannot pass without H^+ so that the ions will enter as ion pairs ($H^+ + A^-$) for which we assume that simultaneous collision with the surface is necessary. Hence the rate of entrance will be proportional to the number of such collisions which is in turn proportional to the product of H_o by A_o. (We omit for the present consideration of exchange of ions of the same sign.)

Just as we consider the permeability of the protoplasm to molecules, P_M, to be equal to the amount of HA entering in unit time through unit surface in molecular form[3] when $M_o - M_i = 1$ so we may consider the permeability of the protoplasm to the ion pair $H^+ + A^-$ to be the amount of HA entering in ionic form ($H^+ + A^-$) in unit time through unit surface when $(H_o)(A_o) - (H_i)(A_i) = 1$ (*i.e.*, when $KM_o - KM_i = 1$). The value of P_A will depend on a "collision" factor G_{H+A} (analogous to G_M) and on a factor D_{H+A} (analogous to D_M) to which we may add a proportionality factor H_{H+A} (analogous to H_M) and write

$$P_A = (H_{H+A})(D_{H+A})(G_{H+A})$$

Letting A_o denote the external and A_i the internal concentration of A^- we may regard the amount entering in unit time at the beginning (when $M_i = 0$ and $A_i = 0$) as $P_A H_o A_o$ which is equal to $P_A K M_o$ (since $H_o A_o = K M_o$).

Hence when $M_i = 0$ and $A_i = 0$ the amount entering in unit time is $P_A K M_o$ and when $M_o = 0$ and $A_o = 0$ the amount passing out in unit time is $P_A K M_i$. When the amounts are expressed as mols it

[14] We shall, for convenience, assume that this is independent of that of molecules. If some ions combine at the surface of the protoplasm to make molecules which pass through the protoplasm in undissociated form and dissociate on the other side it will not affect our calculations except to substitute P_M for P_A.

We neglect for the present any exchange of ions of like sign as this will be taken up later.

is evident that the amount of HA entering in ionic form is equal to the amount of S_i entering so that we may put

$$\frac{dS_i}{dt} = P_A K M_o - P_A K M_i = P_A K (M_o - M_i)$$

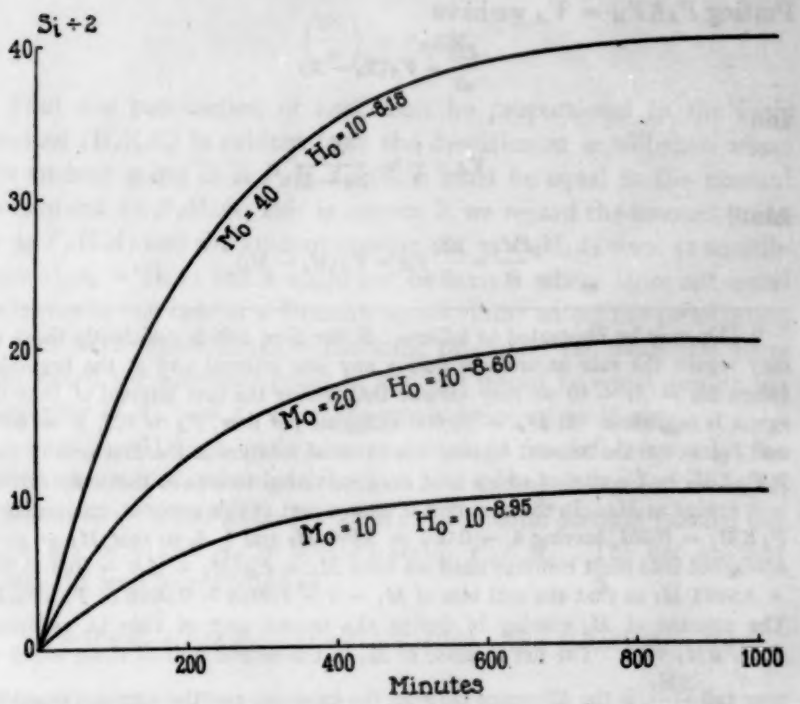

FIG. 3. Calculated time curves of S_i (total amount inside) when ions alone penetrate and F_M and S_o are constant but H_o varies (and in consequence M_o and M_{io} vary). It is assumed that $K = 10^{-8}$, $S_o = 100$, $F_M = 0.5$, and $V_A = 0.001$. The equation is $V_A = \frac{1}{t} \ln_e \frac{S_{io}}{S_{io} - S_i}$.

The same curves would serve if molecules alone entered by putting $V_M = 0.001$ in place of $V_A = 0.001$ or if both molecules and ions entered by putting $V_{MA} = 0.001$ in place of $V_A = 0.001$. In all these cases the rate rises as the external pH value decreases.

(If F_M remains constant the value of $\frac{dS_i}{dt}$ will increase as the external pH value falls and M_o increases, as in Fig. 3.)

Multiplying the right-hand side by $\frac{F_M}{F_M}$ we have

$$\frac{dS_i}{dt} = P_A K F_M \left(\frac{M_o}{F_M} - \frac{M_i}{F_M}\right) = P_A K F_M (S_{ie} - S_i)$$

Putting $P_A K F_M = V_A$ we have

$$\frac{dS_i}{dt} = V_A (S_{ie} - S_i)$$

and

$$V_A = \frac{1}{t} \ln_e \frac{S_{ie}}{S_{ie} - S_i}$$

Also[15]

$$\frac{dM_i}{dt} = \frac{dS_i}{dt} F_M = V_A (M_o - M_i)$$

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[15] This may be illustrated as follows. If the time unit is sufficiently short we may regard the rate as uniform during any one interval and at the beginning (when $M_i = A_i = 0$) we may assume that during the first interval of time the egress is negligible. If $M_o = 10{,}000$ millimols per liter, $P_A = 0.5$, $K = 0.001$ and $F_M = 0.8$ the amount leaving the external solution in the first unit of time is $P_A K M_o = 5$ a part of which is at once converted to ions so that only $(5)(0.8) = 4$ appear as M_i. In the next unit of time a part of this goes out, amounting to $P_A K M_i = 0.002$, leaving $4 - 0.002 = 3.998$ M_i and 1 A_i so that $M_i + A_i = 4.998$, but ions must combine until we have $M_i = F_M(M_i + A_i) = (0.8)(4.998) = 3.9984$ M_i so that the real loss of $M_i = 4 - 3.9984 = 0.0016$ or $P_A K F_M M_i$. The amount of M_i coming in during the second unit of time is, as before, $P_A K F_M M_o = 4$. The net increase of M_i in the second unit of time, which we may call $\frac{\Delta M_i}{dt}$, is the difference between the incoming and the outgoing quantities or $\frac{\Delta M_i}{dt} = P_A K F_M M_o - P_A K F_M M_i = P_A K F_M (M_o - M_i)$ and putting $P_A K F_M = V_A$, the apparent velocity constant of the process, we have

$$\frac{\Delta M_i}{dt} = V_A (M_o - M_i)$$

and for the first two units of time we have

$$V_A = \frac{1}{t} \ln_e \frac{M_o}{M_o - M_i} = \frac{1}{2} \ln_e \frac{10{,}000}{10{,}000 - 7.9984} = 0.0004$$

which agrees with the originally assumed values, *i.e.* $V_A = P_A K F_M = (0.5)(0.001)(0.8) = 0.0004$, whence $P_A = 0.5$ (this value is approximate only; values calculated in this way approach the more closely the assumed value the smaller this value is taken).

and

$$\frac{dA_i}{dt} = \frac{dS_i}{dt} F_A = V_A(S_{ie}F_A - S_{ie}F_A) = V_A(A_{ie} - A_i)$$

and

$$\left(\frac{dS_i}{dt}\right)_b = P_A K M_o$$

That the penetration of ions must be proportional to the ionic product $(H_o)(A_o)$ is evident from the condition at equilibrium where the amount going in is $P_A H_o A_o$ which must be equal to the amount coming out or $P_A H_i A_i$; this is correct if we regard the amount going in as $P_A H_o A_o$ and the amount coming out as $P_A H_i A_i$ since at equilibrium $H_o A_o = H_i A_i$, but it could not be correct where H_i is not equal to H_o (as in the case of a Donnan equilibrium) unless the penetration of ions were proportional to the ionic product. (If molecules alone penetrated the amounts going in and out would be equal despite the inequality of A_{oe} and A_{ie} since M_{oe} would be equal to M_{ie}.)

Case III.—If both molecules and ions go in simultaneously the total amount of S_i passing in (both as ions and as molecules) when $M_i = 0$ and $A_i = 0$ is $P_M M_o + P_A K M_o$. The total amount passing out when $M_o = 0$ and $A_o = 0$ is $P_M M_i + P_A K M_i$. The net amount passing in may be regarded as

$$\frac{dS_i}{dt} = (P_M M_o + P_A K M_o) - (P_M M_i + P_A K M_i) = (P_M + P_A K)(M_o - M_i)$$

When $M_i = A_i = 0$ we may put

$$\left(\frac{dS_i}{dt}\right)_b = (P_M + P_A K) M_o$$

Putting $P_M + P_A K = V$, we have[16]

$$\frac{dS_i}{dt} = V(M_o - M_i) = VF_M\left(\frac{M_{ie}}{F_M} - \frac{M_i}{F_M}\right) = VF_M(S_{ie} - S_i)$$

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[16] Although the velocity constant is VF_M nevertheless for any given value of M_o and M_i the value of $\frac{dS_i}{dt}$ is independent of F_M because when we double F_M we halve S_{ie} since $S_{ie} = \frac{M_o}{F_M}$.

Putting $VF_M = V_{MA}$ we have

$$\frac{dS_i}{dt} = V_{MA}(S_{ie} - S_i)$$

Also[17]

$$\frac{dM_i}{dt} = F_M \frac{dS_i}{dt} = F_M V(M_o - M_i) = V_{MA}(M_o - M_i)$$

and

$$\frac{dA_i}{dt} = F_A \frac{dS_i}{dt} = V_{MA}(S_{ie}F_A - S_iF_A) = V_{MA}(A_{ie} - A_i)$$

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[17] This may be illustrated as follows. Let us put $M_o = 10{,}000$, $P_M = 0.01$, $P_A = 0.5$, $K = 0.001$, $F_M = 0.4$, and $F_A = 0.6$ (we need not assign any value to A_o since it does not enter into our calculations). We then have $V = P_M + P_AK = 0.0105$ and $V_{MA} = VF_M = (0.0105)(0.4) = 0.0042$. At the start when $M_i = A_i = 0$ the molecules entering in the first unit of time $= P_MM_o = (0.01)\ (10{,}000) = 100$ and the amount of A^- entering in ionic form is $P_AKM_o = (0.5)\ (0.001)\ (10{,}000) = 5$, the total being $100 + 5 = 105$ of which $(0.4)\ (105) = 42 = M_i$. During the second unit of time a part of this diffuses out: this part amounts to $[P_MM_i = (0.01)\ (42) = 0.42]$ plus $[P_AKM_i = (0.5)\ (0.001)\ (42) = 0.021]$, the total amount escaping being $0.42 + 0.021 = 0.441$ which must be subtracted from the amount entering in the second unit of time (105) to get the net increase in S_i: this net increase is

$$\frac{\Delta S_i}{dt} = 105 - 0.441 = 104.559$$

of which $M_i = (0.4) \cdot 104.559 = 41.8236$. As a check we may calculate this by the formula given above

$$\frac{\Delta S_i}{dt} = V(M_o - M_i)$$

and get $\frac{\Delta S_i}{dt} = (0.0105)\ (10{,}000 - 42) = 104.559$. Also we may put for the first unit of time

$$\frac{\Delta M_i}{dt} = V_{MA}(M_o - M_i) = (0.0042)\ (10{,}000) = 42$$

We may calculate the value of V_{MA} from the equation

$$V_{MA} = \frac{1}{t} \ln_e \frac{M_i}{M_o - M_i}$$

The correctness of these equations may be tested by considering conditions at equilibrium when the amount coming out is known (since it must be equal to the amount going in). The amount entering in unit time when $M_i = 0$ and $A_i = 0$ may be regarded as $P_M M_o + P_A K M_o$. According to the equations given above[18] the amount coming out when $M_o = 0$ and $A_o = 0$ is $P_M M_i + P_A K M_i$. Hence $P_M M_o + P_A K M_o = P_M M_{io} + P_A K M_{io}$: this is evidently correct since at equilibrium $M_o = M_{io}$.

It is evident that the velocity constant when both ions and molecules enter is equal to the velocity constant when molecules alone enter plus the velocity constant when ions alone enter. We have

(1) Molecules alone entering

$$\frac{dS_i}{dt} = P_M F_M (S_{io} - S_i) = V_M (S_{io} - S_i)$$

(2) Ions alone entering

$$\frac{dS_i}{dt} = P_A F_M K (S_{io} - S_i) = V_A (S_{io} - S_i)$$

(3) Both ions and molecules entering

$$\frac{dS_i}{dt} = (P_M F_M + P_A F_M K)(S_{io} - S_i) = (V_M + V_A)(S_{io} - S_i)$$

Evidently $V_M + V_A = (P_M F_M + P_A F_M K)(S_{io} - S_i) = F_M (P_M + P_A K)(S_{io} - S_i) = V_{MA}$ so that we have

$$\frac{dS_i}{dt} = V_{MA} (S_{io} - S_i)$$

For the first unit of time we have

$$V_{MA} = \ln_e \frac{10{,}000}{10{,}000 - 42} = 0.0042$$

and for the first two units of time

$$V_{MA} = \frac{1}{2} \ln_e \frac{10{,}000}{10{,}000 - (42 + 41.8236)} = 0.0042$$

which agrees with the assumed values $V_{MA} = VF_M = (0.4)(0.01 + 0.005) = 0.0042$. These values are approximate only; values calculated in this way approach nearer to the assumed values the smaller they are taken.

[18] When HA is the only solute present A_o is approximately equal to H_o (except at very low concentrations) so that we may put $(H_o)(A_o) = KM_o = (H_o)^2 = (A_o)^2$.

Since all these velocity constants are functions of F_M they depend on the internal pH value: but they are independent of the external pH value (unless this affects the value of F_M).

Even if the external concentration of HA is kept constant so that the initial diffusion gradient is always the same the value of S_{ie} may vary since at equilibrium $M_e = M_{ie}$ but A_{ie} varies with F_M (the smaller F_M the greater is A_{ie}). If in every case the same amount of HA enters in the first unit of time this will form a smaller fraction of S_{ie} when S_{ie} is large and F_M is small and hence the velocity constant must be small when F_M is small. This statement is based on equations involving S_{ie}, such as $V_M = \frac{1}{t} \ln_e \frac{S_{ie}}{S_{ie} - S_i}$, and is equally true of V_M, V_A, and V_{MA}. It also applies to equations involving M_{ie}, such as $V_M = \frac{1}{t} \ln_e \frac{M_{ie}}{M_{ie} - M_i}$, for if F_M is large the amount of HA entering in the first unit of time will remain largely in the form of M_i and consequently constitute a larger fraction of M_{ie} than when F_M is small: so that the velocity constant V_M must increase with F_M (and this applies equally to V_A and V_{MA}). Similar considerations apply to equations involving A_{ie}, such as $V_M = \frac{1}{t} \ln_e \frac{A_{ie}}{A_{ie} - A_i}$.

Although we have $\frac{dS_i}{dt} = P_M F_M (S_{ie} - S_i)$ this value is independent of F_M since when we double F_M we halve S_{ie} and S_i as is evident from the equation $\frac{dS_i}{dt} = P_M F_M \left(\frac{M_{ie}}{F_M} - \frac{M_i}{F_M}\right)$. This applies also to $\frac{dS_i}{dt}$ in Cases II and III.

If we keep the total outside concentration ($M_e + A_e$) constant and vary the pH value, by adding a base which penetrates slowly or not at all,[19] while the inside pH value remains constant, a decrease in the external pH value will increase the value of $M_e = M_{ie}$ and of $S_{ie} = M_{ie} + A_{ie}$ (*i.e.*, the total inside concentration at equilibrium) and like-

[19] The actual rate can be calculated by means of the equations given later for the penetration of $Na^+ + A^-$.

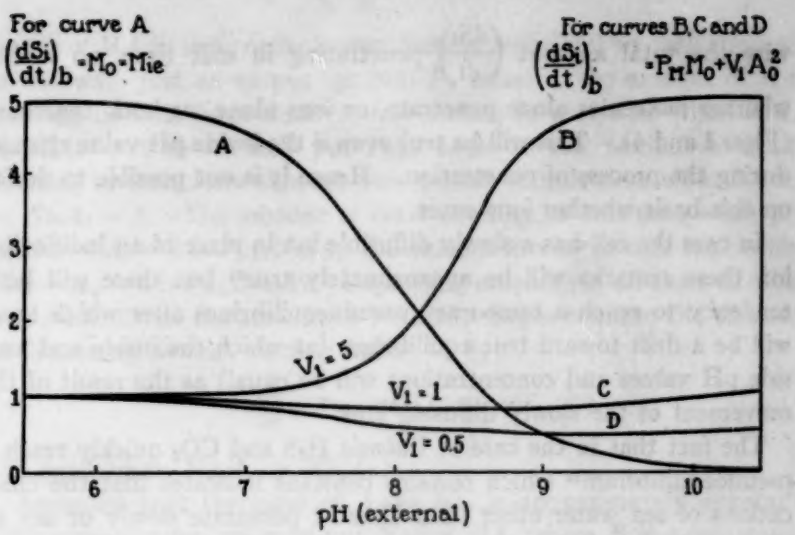

FIG. 4. Curve A, calculated values for the rate of entrance $\left(\frac{dS_i}{dt}\right)_b$ at the start (when $M_i = A_i = 0$). It is assumed that molecules of HA and the ion pair $H^+ + A^-$ penetrate, that $K = 10^{-8}$, $S_o = 5$, F_M is constant and that H_o (and in consequence M_o and M_{ie}) varies. The equation is $\left(\frac{dS_i}{dt}\right)_b = (P_M + P_A K)\, M_o$: where $P_M + P_A K = 1$ this becomes $\left(\frac{dS_i}{dt}\right)_b \times 10^8 = M_o = M_{ie}$.

The same curve would serve if molecules alone entered by putting $P_M = 1$ in place of $P_M + P_A K$ or if ions alone entered by putting $P_A K = 1$ in place of $P_M + P_A K$. In all these cases the initial rate $\left(\frac{dS_i}{dt}\right)_b$ falls off in the same manner as the external pH value increases.

Curves B, C, and D, calculated values for the rate of entrance $\left(\frac{dS_i}{dt}\right)_b$ at the start (when $M_i = A_i = 0$). It is assumed that molecules of HA and the ion pair $Na^+ + A^-$ enter, that $S_o = 1$, F_M is constant, $P_M = 1$, that $Na_o = A_o$, and that V_1 varies (*cf.* Fig. 7). The equation is $\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 A_o^2$ where $V_1 = P_{NaA}$.

When $H_o = H_i$ the velocity constant V_{MNa} behaves somewhat like $\left(\frac{dS_i}{dt}\right)_b$ in Curves B, C, and D: when H_i is constant it behaves like V_{Na} (upper curve) in Fig. 5.

wise the total amount $\left(\frac{dS_i}{dt}\right)$ penetrating in unit time, no matter whether molecules alone penetrate, or ions alone, or both together[20] (Figs. 3 and 4). This will be true even if the inside pH value changes during the process of penetration. Hence it is not possible to decide on this basis whether ions enter.

In case the cell has a slowly diffusible ion in place of an indiffusible ion these remarks will be approximately true[21] but there will be a tendency to reach a temporary pseudoequilibrium after which there will be a drift toward true equilibrium (at which the inside and outside pH values and concentrations will be equal) as the result of the movement of the slowly diffusing ions.

The fact that in the case of *Valonia* H_2S and CO_2 quickly reach a pseudoequilibrium[22] which remains constant indicates that the chief cations of sea water other than H must penetrate slowly or not at all:[23] otherwise A^- would continue to penetrate (paired with some cation other than H^+) until true equilibrium were reached and if this were rapid enough it could be observed experimentally.

Case IV.—If the ion pair $Na^+ + A^-$ alone enters[24] (*i.e.*, the en-

[20] In case both penetrate together the velocity constant will be greater than otherwise, as already shown. In Case III change of H_i may have less effect on the initial rate or the velocity constant.

[21] If Na^+ is inside and comes out very slowly it acts very much like R^+ (in calculations we must bear in mind that it is present on both sides).

[22] It has been shown experimentally for *Valonia* (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–28, **8**, 131; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255) that if a weak acid penetrates rapidly it soon reaches such a pseudoequilibrium. (In the case of *Valonia* the inside pH value is lower than the outside but this does not affect the principle involved.)

[23] The penetration of CO_2 and H_2S does not prove that H^+ penetrates since they may enter in undissociated form.

[24] The penetration of HA will increase and that of $Na^+ + A^-$ will decrease the value of H_i. As an illustration of what is meant we may assume the following values: at the start $H_o = 10^{-5.51855}$, $M_o = 10^{-3.0371}$, $ROH = 10^{-2.99715}$, $R = 10^{-5.498575}$, $H_i = 10^{-5.501425}$, $A_i = 0$, $M_i = 0$. We assume that the dissociation constant for HA is $K = 10^{-8}$ and for $ROH = K_B = 10^{-8}$. After penetration of HA has occurred (the outside being assumed to remain constant) we have $R_{is} = 10^{-4.0371}$, $OH_{is} = 10^{-7}$, $ROH = 10^{-3.0371}$, $H_{is} = 10^{-7}$, $M_{is} = 10^{-3.0371}$, $A_{is} = 10^{-4.0371}$. Let us now add to the outside NaOH and HA until $H_o = 10^{-6}$, A_o

trance of HA in ionic or molecular form is negligible) we may proceed as follows. Just as we put (p. 268) P_A equal to the amount of HA entering in ionic form through unit surface in unit time when $H_oA_o - H_iA_i = 1$ so we may put P_{NaA} equal to the amount of NaA entering in ionic form through unit surface in unit time when $Na_oA_o - Na_iA_i = 1$. The amount of NaA entering in unit time when $M_i = Na_i = A_i = 0$ is $P_{NaA}Na_oA_o$; the amount leaving in unit time when $Na_o = A_o = 0$ is $P_{NaA}Na_iA_i$. Expressing amounts in all cases as mols it is evident that the amount of NaA entering is equal to the amount of S_i entering so that we may write

$$\frac{dS_i}{dt} = P_{NaA}(Na_oA_o - Na_iA_i)$$

(At equilibrium $Na_oA_o = Na_iA_i$.)

Assuming that the ratio of A_i to Na_i is approximately constant during penetration we may put $Na_i = BA_i$ where B is a constant, and for convenience we may put $Na_oA_o = M^2$. We may then write

$$\frac{dS_i}{dt} = P_{NaA}(M^2 - BA_i^2)$$

Assuming that F_A is constant we have (since $A_i = F_AS_i$)

$$\frac{dA_i}{dt} = \frac{dS_i}{dt}F_A = P_{NaA}F_A(M^2 - BA_i^2)$$

On integrating we obtain

$$2P_{NaA}F_AM\sqrt{B} = \frac{1}{t}\ln_e\frac{M + \sqrt{B}A_i}{M - \sqrt{B}A_i}$$

Putting $2P_{NaA}F_AM\sqrt{B} = V_{Na}$ we have

$$V_{Na} = \frac{1}{t}\ln_e\frac{\sqrt{Na_oA_o} + \sqrt{B}A_i}{\sqrt{Na_oA_o} - \sqrt{B}A_i}$$

This gives a curve whose velocity constant, calculated as equal to $\frac{1}{t}\ln_e\frac{A_{ie}}{A_{ie} - A_i}$, increases from the start.[25]

$= 10^{-3}$, $M_o = 10^{-3}$, $Na_o = 10^{-3}$. After penetration we shall have at equilibrium $ROH = 10^{-3}$, $R_{ie} = 10^{-5.002}$, $H_{ie} = 10^{-8.002}$, $A_{ie} = 10^{-2.998}$, $Na_{ie} = 10^{-3.002}$, $M_{ie} = 10^{-3}$ while the outside is assumed to remain unchanged. We see that H_{ie} changes from $10^{-8.5}$ to 10^{-7}.

[25] By way of illustration we may put $A_o = Na_o = 10$, $Na_i = A_i$, $F_A = 0.5$,

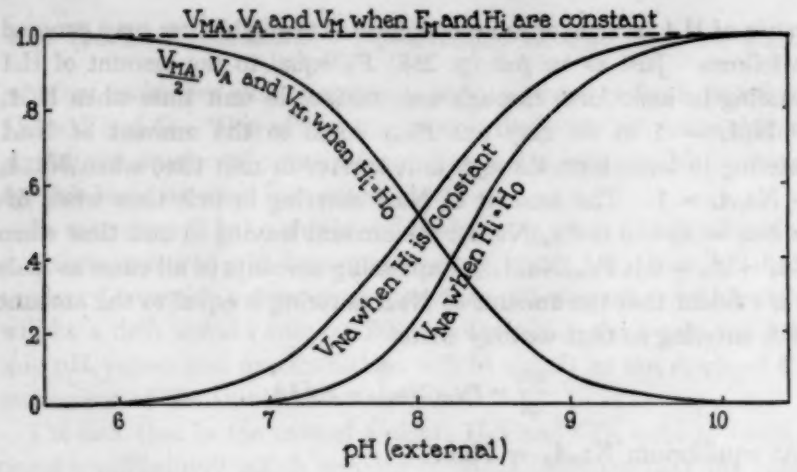

FIG. 5. Shows the change of V_M, V_A, and V_{MA} with change of external pH value (on the supposition that at pH 5.5 $V_M = V_A = V_{MA} = 1$) and also the change of V_{Na} (assuming that its value at pH 10.5 is 1). It is assumed that $S_o = 1$, $K = 10^{-8}$ and $Na_o = A_o$.

As long as F_M remains constant V_M, V_A, and V_{MA} remain constant but if R^+ is so small that H_i is approximately equal to H_o these velocity constants will fall off as the external pH value increases. The equations are $V_M = P_M F_M$; $V_A = P_A K F_M$; $V_{MA} = (P_M + P_A K) F_M$; $V_{Na} = 2P_{NaA} F_A \sqrt{Na_o A_o} \sqrt{B}$.

When H_i is not equal to H_o we put $P_M = P_A K = F_M = 1$ (for V_{MA} we put $F_M = 0.5$) so that $V_M = V_A = V_{MA} = 1$ and when $2P_{NaA} F_A \sqrt{B} = 1$ we have $V_{Na} = A_o$.

When $H_i = H_o$ we have $F_M = M_o$ and may put $V_M = V_A = V_{MA} \div 2 = M_o$. Putting $2P_{NaA} \sqrt{B} = 1$ we have (since $F_A = A_o$) $V_{Na} = A_o^2$.

In general the behavior of V_{MNa} when H_i is constant is like that of V_{Na} when H_i is constant. When $H_i = H_o$ V_{MNa} behaves like $\left(\frac{dSi}{dt}\right)_b$ in Curves *B*, C, and *D* in Fig. 4.

$B = 1$, and $P_{NaA} = 0.01$. Then $V_{Na} = 2P_{NaA} F_A A_o \sqrt{B} = 0.1$. In the first unit of time the total amount S_i entering (when $A_i = Na_i = 0$) is

$$\frac{\Delta S_i}{dt} = P_{NaA} A_o^2 = (0.01)\, 10^2 = 1$$

of which half is A_i since $F_A = 0.5$ so that $A_i = 0.5$ (we assume that none goes out during the first unit of time). In the second unit of time the amount of S_i entering is 1 and the amount going out is $P_{NaA} A_i^2 = 0.01(0.5)^2 = 0.0025$ so that the

We may also write

$$V_{Na} = \frac{1}{t} \ln_e \frac{(\sqrt{Na_o A_o} + \sqrt{B A_i}) \div F_A}{(\sqrt{Na_o A_o} - \sqrt{B A_i}) \div F_A} = \frac{1}{t} \ln_e \frac{(\sqrt{Na_o A_o} + F_A) + \sqrt{B} S_i}{(\sqrt{Na_o A_o} + F_A) - \sqrt{B} S_i}$$

Let us now consider what happens when the total external concentration $S_o (= M_o + A_o)$ is kept constant and the pH value is varied. Let us suppose that at the start HA alone is present and that we add increasing amounts of NaOH. We then have $V_{Na} = 2P_{NaA}F_A \sqrt{Na_o A_o} \sqrt{B}$ and if $Na_o = A_o$ we have $V_{Na} = 2P_{NaA}F_A A_o \sqrt{B}$ so that if F_A is constant V_{Na} is directly proportional to A_o, which increases with increase of external pH value: if R^+ is small so that we can write as an approximation $H_o = H_i$ and if we also put $S_o = 1$ we have $F_A = A_o$. Putting $2P_{NaA}\sqrt{B} = 1$ we have $V_{Na} = F_A A_o = A_o^2$ (see Fig. 5). The initial rate $\left(\frac{dS_i}{dt}\right)_b$ likewise increases with the external pH value since we have

$$\left(\frac{dS_i}{dt}\right)_b = P_{NaA} Na_o A_o$$

and putting $P_{NaA} = V_1$ this becomes

$$\left(\frac{dS_i}{dt}\right)_b = V_1 Na_o A_o$$

Then

$$\left(\frac{dA_i}{dt}\right)_b = F_A \left(\frac{dS_i}{dt}\right)_b = V_1 F_A Na_o A_o$$

Putting $V_1 F_A = V_2$ we have

$$\left(\frac{dA_i}{dt}\right)_b = V_2 Na_o A_o$$

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net increase in S_i is $1 - 0.0025 = 0.9975$ of which half or 0.49875 is A_i. Hence at the end of the second period $A_i = 0.5 + 0.49875 = 0.99875$ and

$$V_{Na} = \frac{1}{t} \ln_e \frac{10 + 0.99875}{10 - 0.99875} = 0.1$$

This is approximate (in all such cases the approximation is nearer the smaller the value of V_{Na}).

Case V.—Molecules of HA *and the Ion Pair* $Na^+ + A^-$ *Enter.* We then have

$$\frac{dA_i}{dt} = V_M(A_{ie} - A_i) + V_2(Na_oA_o - Na_iA_i)$$

If the proportion of Na_i to A_i remains sufficiently constant during penetration we may write as an approximation $Na_i = BA_i$. Putting $V_MA_{ie} + V_2Na_oA_o = E$ we have

$$\frac{dA_i}{dt} = E - V_MA_i - V_2BA_i^2$$

On integrating (between 0 and A_{ie}) we obtain

$$\sqrt{V_M^2 + 4EV_2B} = \frac{1}{t}\ln_e \frac{\sqrt{V_M^2 + 4EV_2B} + 2V_2BA_i + V_M}{\sqrt{V_M^2 + 4EV_2B} - 2V_2BA_i - V_M}\left(\frac{\sqrt{V_M^2 + 4EV_2B} - V_M}{\sqrt{V_M^2 + 4EV_2B} + V_M}\right)$$

Putting $\sqrt{V_M^2 + 4EV_2B} = V_{MNa}$ and $2V_2B = V_3$ we have

$$V_{MNa} = \frac{1}{t}\ln_e \frac{V_{MNa} + V_3A_i + V_M}{V_{MNa} - V_3A_i - V_M}\left(\frac{V_{MNa} - V_M}{V_{MNa} + V_M}\right)$$

Putting $V_{MNa} + V_M = V_4$ and $V_{MNa} - V_M = V_5$ we have[26]

$$V_{MNa} = \frac{1}{t}\ln_e \frac{V_4 + V_3A_i}{V_5 - V_3A_i}\left(\frac{V_5}{V_4}\right)$$

---

[26] As an illustration we may put $M_o = M_{ie} = 22.2$, $A_o = Na_o = 10$, $A_{ie} = 11.1$, $F_M = 0.667$, $F_A = 0.333$, $B = 0.811$, $P_M = 0.0015$, $P_{Na,A} = 0.003$, $V_M = P_MF_M = 0.001$, and $V_2 = P_{Na,A}F_A = 0.001$. In the first unit of time the amount going in is

$$\frac{\Delta S_i}{dt} = P_MM_o + P_{Na,A}Na_oA_o = (0.0015)22.2 + (0.003)(10)(10) = 0.3333$$

We then have $A_i = F_AS_i = (0.333)(0.3333) = 0.1111$ and $M_i = F_MS_i = 0.2222$. We may assume for convenience that none of this goes out during the first unit of time but that during the second unit of time the amount going out is $P_MM_i + P_{Na,A}Na_iA_i = P_MM_i + P_{Na,A}BA_i^2 = (0.0015)(0.222) + (0.003)(0.811)(0.1111)^2 = 0.00036291$. This must be subtracted from the total amount going in during the first two units of time (which is $2(0.3333) = 0.6666$) to get the net total inside at the end of the second unit of time: this is $0.6666 - 0.00036291 = 0.666237$ of which 1/3 or 0.222079 is A_i. We may arrive at the same result by using the formula

$$\frac{\Delta A_i}{dt} = V_M(A_{ie} - A_i) + V_2(Na_oA_o - BA_i^2)$$

This gives a curve whose velocity constant calculated as equal to $\frac{1}{t} \ln_e \frac{A_{i_e}}{A_{i_e} - A_i}$ increases from the start.

Let us now consider the significance of V_{MNa}. We have $V_{MNa} = \sqrt{V_M^2 + 4EV_2B}$. Substituting the value of E and putting $Na_e = A_e$ we have

$$V_{MNa} = \sqrt{V_M^2 + 4V_MV_2BA_{i_e} + 4V_2^2BA_e^2}$$

If R^+ is small we may write as an approximation $B = 1$ and $A_e = A_{i_e}$. We then have

$$\begin{aligned} V_{MNa} &= \sqrt{V_M^2 + 4V_MV_2A_e + 4V_2^2A_e^2} \\ &= V_M + 2V_2A_e \\ &= P_MF_M + 2P_{Na,A}F_AA_e \end{aligned}$$

so that in case F_M and F_A are approximately constant V_{MNa} will increase as A_e increases, *i.e.*, with increase of external pH value.[37]

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and putting $A_i = 0$ at the start. We then have for the first unit of time

$$\frac{\Delta A_i}{dt} = V_MA_{i_e} + V_2Na_eA_e$$

or $(0.001)(11.1) + (0.001)(10)(10) = 0.1111$. At the end of the first unit of time we have $A_i = 0.1111$ and for the second unit of time

$$\frac{\Delta A_i}{dt} = V_M(A_{i_e} - A_i) + V_2(Na_eA_e - BA_i) = (0.001)(11.1 - 0.1111) +$$

$$(0.001)\,[100 - (0.811)\,(0.1111)^2] = 0.110979$$

adding this to the amount of A_i present at the beginning of the second unit of time we have $0.1111 + 0.110979 = 0.222079\ A_i$.

For the first unit of time we have

$$t = \frac{1}{V_{MNa}} \ln_e \frac{V_i + V_2A_i}{V_i - V_2A_i}\left(\frac{V_i}{V_i}\right) = \frac{1}{0.019} \ln_e \frac{0.020 + 0.001622\,(0.1111)}{0.018 - 0.001622\,(0.1111)}\left(\frac{0.018}{0.020}\right) = 1$$

For the first two units of time taken together we have

$$t = \frac{1}{0.019} \ln_e \frac{0.020 + 0.001622\,(0.222079)}{0.018 - 0.001622\,(0.222079)}\left(\frac{0.018}{0.020}\right) = 2$$

These values are approximate: they approach nearer to the assumed values of t the smaller the values assumed for V_M and V_2.

[37] This would also be true if we did not assume that $A_e = A_{i_e}$ or that $Na_e = A_e$ since we should have $V_{MNa} = \sqrt{\text{constant} + Na_eA_e}$.

But if the internal pH value rises with the external F_M will fall off. If R^+ is small we may write as an approximation $H_o = H_{io}$. If H_i does not change greatly during penetration and if $S_o = M_o + A_o = 1$ we have $F_M = M_o$ and $F_A = A_o$. We then have $V_{MNa} = P_M M_o + P_{NaA} A_o^2$ so that the behavior of V_{MNa} will resemble that of $\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 A_o^2 = P_M M_o + P_{NaA} A_o^2$ in curves B, C, and D in Fig. 4. Hence if the value of P_{NaA} is small in comparison with that of P_M we shall expect V_{MNa} to fall off as the external pH value rises but if the value of P_{NaA} is large in comparison with that of P_M we shall expect V_{MNa} to increase as the external pH value increases.

We also have

$$\frac{dS_i}{dt} = \frac{dA_i}{dt}\left(\frac{1}{F_A}\right) = (E - V_M A_i - V_2 B A_i^2) \div F_A$$

and

$$V_{MNa} = \frac{1}{t} \ln_e \frac{V_4 + F_A + V_3 S_i}{V_3 + F_A - V_3 S_i}\left(\frac{V_3}{V_4}\right)$$

and

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 \mathrm{Na}_o A_o$$

Let us now consider the behavior of $\left(\frac{dS_i}{dt}\right)_b$ with increase in external pH value. In the beginning when $M_i = A_i = 0$ the amount going in as molecules in unit time is $P_M M_o$ and if $\mathrm{Na}_o = A_o$ the amount of S_i entering in ionic form is $V_1 \mathrm{Na}_o A_o = V_1 A_o^2$. If we put $P_M = V_1 = 1$, keep the total external concentration ($M_o + A_o = S_o$) constant, and change the dissociation of HA by adding various quantities of NaOH we may calculate the total amount entering, *i.e.*,

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 A_o^2 = M_o + A_o^2$$

for each value of M_o.[28] Fig. 6 shows the results of such calculations

[28] *E.g.*, if we put $P_M = V_2 = S_o = 1$, $M_o = 0.5$ (*i.e.*, $M_o = 50$ per cent), and $A_o = 0.5$, we have

$$\left(\frac{\Delta S_i}{dt}\right)_b = 0.5 + (0.5)^2 = 0.75$$

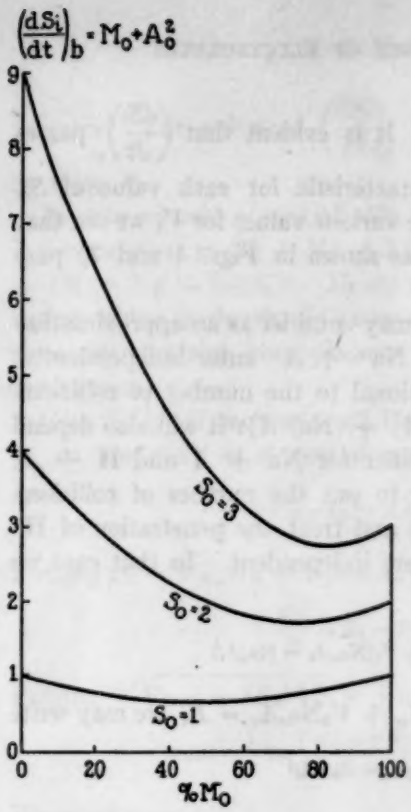

FIG. 6

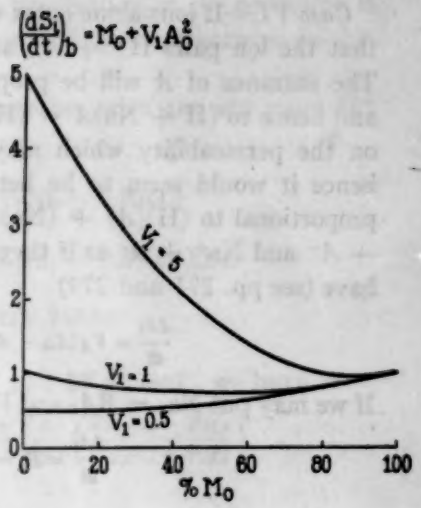

FIG. 7

FIG. 6. Calculated values of $\left(\frac{dS_i}{dt}\right)_b$ = initial rate of entrance (when $M_i = A_i = 0$) if molecules of HA and the ion pair $Na^+ + A^-$ enter. The equation is

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + P_{NaA} Na_o A_o$$

We assume that $Na_o = A_o$ and that $P_M = P_{NaA} = 1$. We then have

$$\left(\frac{dS_i}{dt}\right)_b = M_o + A_o^2$$

FIG. 7. Calculated values of $\left(\frac{dS_o}{dt}\right)_b$ = initial rate of entrance (when $M_i + A_i = 0$) if molecules of HA and the ion pair $Na^+ + A^-$ enter. The equation is

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + V_1 Na_o A_o$$

where $V_1 = P_{NaA}$. We assume that $Na_o = A_o$, $P_M = S_o = 1$, but V_1 varies. When V_1 is small the curves do not pass through a marked minimum.

for $S_o = 1$, $S_o = 2$, and $S_o = 3$. It is evident that $\left(\frac{dS_i}{dt}\right)_b$ passes through a minimum which is characteristic for each value of S_o. If we put $S_o = P_M = 1$ and assume various values for V_1 we see that above a certain value the curves (as shown in Figs. 4 and 7) pass through a minimum.

Case VI.—If ions alone enter we may consider as an approximation that the ion pairs $H^+ + A^-$ and $Na^+ + A^-$ enter independently. The entrance of A will be proportional to the number of collisions and hence to $(H + Na)A = (H)(A) + (Na)(A)$: it will also depend on the permeability which may differ for $Na + A$ and $H + A$, hence it would seem to be better to put the number of collisions proportional to $(H)(A) + (Na)(A)$ and treat the penetration of $H^+ + A^-$ and $Na^+ + A^-$ as if they were independent. In that case we have (see pp. 271 and 279)

$$\frac{dA_i}{dt} = V_A(A_{io} - A_i) + V_2(Na_oA_o - Na_iA_i)$$

If we may put $Na_i = BA_i$ and $V_AA_{io} + V_2Na_oA_o = E_1$ we may write

$$\frac{dA_i}{dt} = E_1 - V_AA_i - V_2BA_i^2$$

On integrating (between 0 and A_{io}) and putting $\sqrt{V_A^2 + 4E_1V_2B} = V_{AN_a}$ and $2V_2B = V_2$ we have

$$V_{AN_a} = \frac{1}{t}\ln_e \frac{V_{AN_a} + V_2A_i + V_A}{V_{AN_a} - V_2A_i - V_A}\left(\frac{V_{AN_a} - V_A}{V_{AN_a} + V_A}\right)$$

We also have

$$\frac{dS_i}{dt} = \frac{dA_i}{dt}\left(\frac{1}{F_A}\right) = (E_1 - V_AA_i - V_2BA_i^2) \div F_A$$

and

$$V_{AN_a} = \frac{1}{t}\ln_e \frac{(V_{AN_a} + V_A) \div F_A + V_2S_i}{(V_{AN_a} - V_A) \div F_A - V_2S_i}\left(\frac{V_{AN_a} - V_A}{V_{AN_a} + V_A}\right)$$

$$\left(\frac{dS_i}{dt}\right)_b = P_AH_oA_o + V_1Na_oA_o = P_AKM_o + V_1Na_oA_o$$

It is evident that if S_o remains constant and the external pH value increases H_oA_o will decrease (since $H_oA_o = KM_o$) but Na_oA_o will

increase. The value of $\left(\frac{dS_i}{dt}\right)_b$ will pass through a minimum as in Case V.

In case we add a neutral salt, *e.g.*, LiCl, the number of collisions is proportional to (H + Na + Li) (*A* + Cl) = (H + Na + Li)(*A*) + (H + Na + Li)(Cl). Hence the addition of a neutral salt whose cation enters freely should favor the penetration of A^- providing it does not diminish protoplasmic permeability or otherwise inhibit the process.

Case VII.—If molecules of H*A* together with the ion pairs $H^+ + A^-$ and $Na^+ + A^-$ enter we have

$$\frac{dA_i}{dt} = V_{MA}(A_{io} - A_i) + V_2(Na_oA_o - Na_iA_i)$$

Putting $E_2 = V_{MA}A_{io} + V_2Na_oA_o$ and $Na_i = BA_i$ we have

$$\frac{dA_i}{dt} = E_2 - V_{MA}A_i - V_2BA_i^2$$

Putting $\sqrt{V_{MA}^2 + 4E_2V_2B} = V_{MAN_a}$ and $2V_2B = V_3$ we have

$$V_{MAN_a} = \frac{1}{t}\ln_e \frac{V_{MAN_a} + V_3A_i + V_{MA}}{V_{MAN_a} - V_3A_i - V_{MA}}\left(\frac{V_{MAN_a} - V_{MA}}{V_{MAN_a} + V_{MA}}\right)$$

We also have

$$\frac{dS_i}{dt} = (E_2 - V_{MA}A_i - V_2BA_i^2) + F_A$$

and

$$V_{MAN_a} = \frac{1}{t}\ln_e \frac{(V_{MAN_a} + V_{MA}) + F_A + V_3S_i}{(V_{MAN_a} - V_{MA}) + F_A - V_3S_i}\left(\frac{V_{MAN_a} - V_{MA}}{V_{MAN_a} + V_{MA}}\right)$$

also $$\left(\frac{dS_i}{dt}\right)_b = (P_M + P_AK)M_o + V_1Na_oA_o$$

The value of $\left(\frac{dS_i}{dt}\right)_b$ will pass through a minimum as in Case V.

Case VIII.—If a weak acid H*A* and a weak base *Z*OH enter simultaneously, forming the salt *ZA* inside, we may use for H*A* and for *Z*OH the equations already given (which enable us in some cases to find the amount of H*A*, H^+, A^-, *Z*OH, and Z^+ inside at any given moment).

If the dissociation constant, K, of HA is equal to the dissociation constant, K_Z, of ZOH and if HA and ZOH enter in equal amounts the internal pH value will remain almost constant and in consequence F_M and F_{MZ} will remain almost constant (F_{MZ}, the fractional concentration of molecules of ZOH inside $= \frac{M_{Zi}}{M_{Zi} + Z_i}$, where M_{Zi} is the concentration of molecules of ZOH inside and Z_i is the concentration of Z^+ inside).

It is evident from what has been said that when a weak electrolyte such as HA is the only solute penetrating we cannot decide whether ions enter by comparing the rates or the velocity constants at high and low pH values for the relative rates will change in the same way in both cases (*i.e.*, whether molecules alone or ions alone penetrate) and the velocity constants, although differing in the two cases, will act alike. If on the other hand the molecules of a weak electrolyte, HA, and the ions of a salt NaA penetrate together we shall get for $\left(\frac{dS_i}{dt}\right)_b$ a curve like Curve A in Fig. 4 if molecules alone (or ions alone) of HA penetrate, but if the ion pair $Na^+ + A^-$ penetrates in addition to molecules of HA we may expect the types shown in Curves B, C, and D in Fig. 4, as well as those shown in Figs. 6 and 7. The velocity constants will also differ as shown in Fig. 5. If molecules alone (or ions alone) of HA penetrate the velocity constant will remain constant if H_i remains constant, but if H_i approximates H_o the velocity constant will fall off as the external pH value rises; on the other hand the velocity constant will increase with external pH value when the ion pair $Na^+ + A^-$ alone enters. If both molecules of HA and the ion pair $Na^+ + A^-$ enter the velocity constant V_{MNa} may increase or decrease as the case may be. When F_M is constant V_{MNa} acts much like V_{Na} in Fig. 5 (upper curve) but when $H_o = H_i$ it acts much like $\left(\frac{dS_i}{dt}\right)_b$ in Curves B, C, and D in Fig. 4.

In case of an exchange of ions[29] of the same sign going in opposite directions, *e. g.*, exchange of H^+ for Na^+, the total quantity of cations,

[29] Doubtless the cell can produce sufficient ions (*e. g.*, $H^+ + HCO_3^-$) to ensure adequate exchange.

Q, passing inward through the protoplasm in unit time is proportional to the total concentration (H + Na) of cations inside multiplied by that outside so that we may put $Q = L(H_i + Na_i)(H_o + Na_o)$ where L is a proportionality factor and the subscripts i and o denote the concentrations inside and outside respectively (Q will depend on the rate of passage of the slower cation). The quantity of H^+ passing out is equal to $\frac{Q H_i}{H_i + Na_i}$ and that passing in is equal to $\frac{Q H_o}{H_o + Na_o}$ and the net amount passing in $\left(\frac{dH}{dt}\right)$ is the difference between these expressions: on reducing them to a common denominator we get

$$\frac{dH}{dt} = \frac{Q}{(H_o + Na_o)(H_i + Na_i)}(H_oNa_i - H_iNa_o)$$

Now $H_o + Na_o = H_{ob}$ (the concentration of H_o at the start) and $H_i + Na_i = Na_{ib}$ (the concentration of Na_i at the start). Putting $\frac{Q}{H_{ob}Na_{ib}} = U$ we have

$$\frac{dH}{dt} = U(H_oNa_i - H_iNa_o)$$

It is, however, more convenient to proceed as follows. We put

$$\frac{dH}{dt} = \frac{QH_o}{H_o + Na_o} - \frac{QH_i}{H_i + Na_i}$$

$$= \frac{QH_o}{H_{ob}} - \frac{QH_i}{Na_{ib}}$$

$$= U(H_oNa_{ib} - H_iH_{ob})$$

$$= UNa_{ib}\left(\frac{H_oNa_{ib}}{Na_{ib}} - \frac{H_iH_{ob}}{Na_{ib}}\right) = UNa_{ib}\left(H_o - \frac{H_{ob}}{Na_{ib}}H_i\right)$$

Putting $UNa_{ib} = U_1$ and $\frac{H_{ob}}{Na_{ib}} = U_2$ we have

$$\frac{dH}{dt} = U_1(H_o - U_2H_i)$$

If the external solution is relatively large or the concentration of H_o relatively large, so that we may regard H_o as constant, we may put

$$U_1 = \frac{1}{tU_2} \ln_e \frac{H_o}{H_o - U_2 H_i}$$

Experimental tests of these equations are in progress. It may be noted that if we regard the free base of the basic dye brilliant cresyl blue as undissociated we should expect the velocity constant V_M of diffusion into a living cell of *Nitella* to be constant (*cf.* Fig. 5) as long as the pH value of the sap is constant despite the fact that the external pH value changes. This is the result actually found by Irwin.[30]

Let us now consider briefly certain complications due to the fact that the penetrating substance may have different activities in the protoplasm and the aqueous solutions. Overton states that permeability depends largely on the partition coefficient between the outer surface of the protoplasm and the external solution and according to Irwin[31] the partition coefficient at the internal surface is also important.

The equation $\frac{dM_i}{dt} = V_M(M_o - M_i)$ assumes that the partition coefficient between the protoplasm and the external and internal solutions is 1. In case it differs from 1 and is the same for both surfaces of the protoplasm we may call it K_o. If we follow the treatment of Northrop[32] we may put $\frac{dM_i}{dt} = V_M(K_oM_o - K_oM_i) = V_MK_o(M_o - M_i)$. In case it is K_o for the external and K_i for the internal surface[33] (due to a difference between the external solution and the sap) we may put $\frac{dM_i}{dt} = V_M(K_oM_o - K_iM_i)$,[1] whence

$$\frac{dM_i}{dt} = V_MK_o\left(\frac{K_oM_o}{K_o} - \frac{K_iM_i}{K_o}\right).$$

[30] Irwin, M., *J. Gen. Physiol.*, 1922–23, **5**, 727.

[31] Irwin, M., *J. Gen. Physiol.*, 1928–29, **12**, 407.

[32] Northrop, J. H., *J. Gen. Physiol.*, 1928–29, **12**, 435.

[33] For convenience we here regard the protoplasm as a homogeneous non-aqueous phase separating the external solution from the sap.

Putting $\frac{K_i}{K_o} = K_{io}$ and $V_M K_o = V_{M_o}$ we have

$$\frac{dM_i}{dt} = V_{M_o}(M_o - K_{io}M_i)$$

or

$$V_{M_o} = \frac{1}{tK_{io}} \ln_e \frac{M_o}{M_o - K_{io}M_i}$$

For Case II we may proceed as follows. The concentration of molecules in the outer surface of the protoplasm may be called M_{po} and that in the inner surface M_{pi}. If the dissociation constant is K_p we have $H_{po}A_{po} = K_p M_{po}$ and $H_{pi}A_{pi} = K_p M_{pi}$, where the subscripts *po* and *pi* signify concentrations in the outer and inner surfaces respectively. The diffusion gradient for the ion pair $H^+ + A^-$ will therefore be proportional to $H_{po}A_{po} - H_{pi}A_{pi}$ or to $K_p M_{po} - K_p M_{pi}$ which is equal to $K_p K_o M_o - K_p K_i M_i$. In place of the equation already given for Case II, *i.e.* $\frac{dM_i}{dt} = V_A(M_o - M_i)$ we then have

$$\frac{dM_i}{dt} = V_A (K_p K_o M_o - K_p K_i M_i)$$

Putting $K_p K_o = K'_o$ and $K_p K_i = K'_i$ we have

$$\frac{dM_i}{dt} = V_A(K'_o M_o - K'_i M_i)$$

and proceeding as before we obtain

$$\frac{dM_i}{dt} = V_A K'_o (M_o - K'_{io} M_i)$$

where $K'_{io} = \frac{K'_i}{K'_o}$. Putting $V_A K'_o = V'_A$ we have

$$V'_A = \frac{1}{tK'_{io}} \ln_e \frac{M_o}{M_o - K'_{io}M_i}.$$

Case III is simply the combination of the equations and presents no difficulty.

For Case IV we may proceed as in Case II. Assuming for purposes of calculation a few undissociated molecules of NaA we may call the dissociation constant of NaA in the protoplasm K'_p. The diffusion gradient is then proportional to $\mathrm{Na}_{po}A_{po} - \mathrm{Na}_{pi}A_{pi} = K'_pK''_oM_o - K'_pK''_iM_i$ where K''_o and K''_i are the partition coefficients for NaA at the outer and inner surfaces and M'_o and M'_i are the concentrations of undissociated molecules of NaA. In place of the equation already given for Case IV, *i.e.* $\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_A(\mathrm{Na}_oA_o - \mathrm{Na}_iA_i)$ we have

$$\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_A(\mathrm{Na}_{po}A_{po} - \mathrm{Na}_{pi}A_{pi})$$

The relation between $(\mathrm{Na}_{po}A_{po})$ and (Na_oA_o) is obtained as follows

$$K''_o = \frac{M'_{po}}{M'_o} = \frac{\mathrm{Na}_{po}A_{po} + K'_p}{\mathrm{Na}_oA_o + K_e}$$

where K_e is the dissociation constant of NaA in the external solution. From this we obtain $\frac{\mathrm{Na}_{po}A_{po}}{\mathrm{Na}_oA_o} = \frac{K''_oK'_p}{K_e}$. We may put $\frac{K''_oK'_p}{K_e} = K'''_o$ and call the corresponding constant for the inner surface K'''_i. We then have

$$\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_A(K'''_o\mathrm{Na}_oA_o - K'''_i\mathrm{Na}_iA_i)$$

$$= P_{\mathrm{Na}A}F_AK'''_o(\mathrm{Na}_oA_o - K'''_{io}\mathrm{Na}_iA_i)$$

where $K'''_{io} = \frac{K'''_i}{K'''_o}$. Putting $M^2 = \mathrm{Na}A_o$, $\mathrm{Na}_i = BA_i$, and $K'''_{io}B = B'$ we have

$$\frac{dA_i}{dt} = P_{\mathrm{Na}A}F_AK'''_o(M^2 - B'A_i^2)$$

or

$$2P_{\mathrm{Na}A}F_AM\sqrt{B'} = \frac{1}{t}\ln_e\frac{M + \sqrt{B'}A_i}{M - \sqrt{B'}A_i}$$

Putting $2P_{Na}F_AM\sqrt{B'} = V'_{Na}$ we have

$$V_{Na} = \frac{1}{t} \ln_e \frac{\sqrt{Na_oA_o} + \sqrt{B'}A_t}{\sqrt{Na_oA_o} - \sqrt{B'}A_t}$$

Cases V, VI, VII, and VIII are merely combinations of the equations already considered and hence present no difficulty; they involve only a change of constants.

Hitherto we have treated only those cases where the time curve is of the first order but in practice we may find when we calculate according to the equation $K = \frac{1}{t} \ln_e \frac{a}{a-x}$ that the value of K falls off with time. This might suggest that the curve follows a dimolecular equation but in that case we should expect that on taking any two curves, which we may call I and II, and multiplying all the ordinates of Curve I by a factor to make the final (equilibrium) value equal that of Curve II the two curves would not coincide. For if they do coincide it means that their ordinates at any given time t must bear the same relation as their final equilibrium values. If we are dealing with irreversible reactions the final or equilibrium value of the amount transformed, which we may call x_e, is equal to the amount present at the start, which we may call a. If a monomolecular process giving Curve I M is changed to a monomolecular process giving Curve II M, by simply doubling the amount of a the velocity constant is not changed and the time Curve II M can be reduced to Curve I M by dividing all its ordinates by 2. But if we have a dimolecular process giving Curve I D with the equation $\frac{dx}{dt} = K(a-x)^2$ or $K = \frac{1}{t} \frac{x}{a(a-x)}$ we cannot double the amount of a without changing the velocity constant and Curve II D cannot be reduced to I D by dividing its ordinates by 2. Hence when we have a set of curves with different values of a which are convertible into each other simply by multiplying ordinates we may conclude that they are not of higher orders than the first. Such curves are like those of a reaction of the first order in which the velocity constant diminishes from the start and may for con-

venience be called "inhibited curves." They may often be fitted by empirical formulae such as

$$K = \frac{1}{t} \ln_e \frac{a}{a - bx^n}$$

or

$$K = \frac{ba}{tx^n} \ln_e \left(\frac{a}{a-x}\right) - \frac{b}{x^n}$$

or

$$K = \frac{a}{b^2 t} \ln_e \frac{a}{a - bx} - \frac{x}{b}$$

Similar considerations apply to the exit of electrolytes but in practice there may be complications, such, for example, as those discussed by Irwin.[34]

What is here said of weak acids holds, with suitable modifications, for weak bases and for amphoteric electrolytes, and it may also be applied to strong electrolytes since it is always permissible to assume the existence of a few undissociated molecules for purposes of calculation.

SUMMARY

When the only solute present is a weak acid, HA, which penetrates as molecules only into a living cell according to a curve of the first order and eventually reaches a true equilibrium we may regard the rate of increase of molecules inside as

$$\frac{dM_i}{dt} = P_M F_M (M_o - M_i)$$

where P_M is the permeability of the protoplasm to molecules, M_o denotes the external and M_i the internal concentration of molecules, A_i denotes the internal concentration of the anion A^- and $F_M = \frac{M_i}{M_i + A_i}$. (It is assumed that the activity coefficients equal 1.) Putting $P_M F_M = V_M$, the apparent velocity constant of the process, we have

$$\frac{dM_i}{dt} = V_M(M_o - M_i) = V_M(M_{io} - M_i)$$

[34] Irwin, M., *J. Gen. Physiol.*, 1926–27, **10**, 75.

where e denotes the concentration at equilibrium. Then

$$V_M = \frac{1}{t} \ln_e \frac{M_{ie}}{M_{ie} - M_i}$$

where t is time.

The corresponding equation when ions alone enter is

$$\frac{dA_i}{dt} = P_A K F_M (A_{ie} - A_i)$$

where K is the dissociation constant of HA, P_A is the permeability of the protoplasm to the ion pair $H^+ + A^-$, and A_{ie} denotes the internal concentration of A_i at equilibrium. Putting $P_A K F_M = V_A$, the apparent velocity constant of the process, we have

$$\frac{dA_i}{dt} = V_A (A_{ie} - A_i)$$

and

$$V_A = \frac{1}{t} \ln_e \frac{A_{ie}}{A_{ie} - A_i}$$

When both ions and molecules of HA enter together we have

$$\frac{dS_i}{dt} = (V_M + V_A)(S_{ie} - S_i) = V_{MA}(S_{ie} - S_i)$$

where $S_i = M_i + A_i$ and S_{ie} is the value of S_i at equilibrium. Then

$$V_{MA} = \frac{1}{t} \ln_e \frac{S_{ie}}{S_{ie} - S_i}$$

V_M, V_A, and V_{MA} depend on F_M and hence on the internal pH value but are independent of the external pH value except as it affects the internal pH value.

When the ion pair $Na^+ + A^-$ penetrates and $Na_i = BA_i$ we have

$$\frac{dA_i}{dt} = P_{NaA} F_A (Na_e A_e - BA_i^2)$$

and

$$V_{Na} = 2P_{NaA} F_A \sqrt{Na_e A_e B} = \frac{1}{t} \ln_e \frac{\sqrt{Na_e A_e} + \sqrt{BA_i}}{\sqrt{Na_e A_e} - \sqrt{BA_i}}$$

where P_{NaA} is the permeability of the protoplasm to the ion pair $Na^+ + A^-$, Na_e and Na_i are the external and internal concentra-

tions of Na^+, $F_A = \frac{A_i}{S_i}$, $B = \frac{Na_i}{A_i}$, and V_{Na} is the apparent velocity constant of the process.

Equations are also given for the penetration of:

(1) molecules of HA and the ion pair $Na^+ + A^-$,

(2) the ion pairs $H^+ + A^-$ and $Na^+ + A^-$,

(3) molecules of HA and the ion pairs $Na^+ + A^-$ and $H^+ + A^-$.

(4) The penetration of molecules of HA together with those of a weak base ZOH.

(5) Exchange of ions of the same sign.

When a weak electrolyte HA is the only solute present we cannot decide whether molecules alone or molecules and ions enter by comparing the velocity constants at different pH values, since in both cases they will behave alike, remaining constant if F_M is constant and falling off with increase of external pH value if F_M falls off. But if a salt (*e.g.*, NaA) is the only substance penetrating the velocity constant will increase with increase of external pH value: if molecules of HA and the ions of a salt NaA penetrate together the velocity constant may increase or decrease while the internal pH value rises.

The initial rate $\left(\frac{dS_i}{dt}\right)_0$ (*i.e.*, the rate when $M_i = 0$ and $A_i = 0$) falls off with increase of external pH value if HA alone is present and penetrates as molecules or as ions (or in both forms). But if a salt (*e.g.*, NaA) penetrates the initial rate may in some cases decrease and then increase as the external pH value increases.

At equilibrium the value of M_i equals that of M_e (no matter whether molecules alone penetrate, or ions alone, or both together). If the total external concentration ($S_e = M_e + A_e$) be kept constant a decrease in the external pH value will increase the value of M_e and make a corresponding increase in the rate of entrance and in the value at equilibrium no matter whether molecules alone penetrate, or ions alone, or both together.

What is here said of weak acids holds with suitable modifications for weak bases and for amphoteric electrolytes and may also be applied to strong electrolytes.

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THE SUBSTRATE IN PEPTIC SYNTHESIS OF PROTEIN

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(Accepted for publication, October 16, 1929)

Pepsin exhibits in its proteolytic action a certain, though limited, specificity. Certain protamines for example are not attacked, and it does not hydrolyze the products of its own action on protein. Similarly it appears to exhibit a limited specificity in its synthetic action. Resynthesis is not obtained with all concentrated peptic digests.

The facts that the same number of amino and carboxyl groups are liberated in peptic hydrolysis (1), and that these groups are increased in equal amount also in tryptic and in ereptic hydrolysis, suggest that the specificity exhibited by these hydrolytic enzymes is associated not with the amide linkage *per se*, but with a particular configuration of the molecule attacked.

The possibility that it is the presence or absence of an analogous complex which determines the synthesizeability of a given mixture of protein cleavage products is suggested by the following observations which are described in detail later. It was found that the yield of protein that could be synthesized from a given peptic digest was highest when the hydrolyzing action of the pepsin was stopped as soon as all the protein had disappeared from the solution. The yield grew less the longer the digest was permitted to contain active enzyme after this time. A possible explanation for this progressive diminution in the yield of protein synthesized, with the length of time which is allowed to elapse before the digest is prepared for synthesis, *i.e.* before it is concentrated at pH 4.0 after the pepsin is destroyed by boiling, is that the hypothetical complex essential for peptic synthesis is hydrolyzed in the course of a secondary hydrolysis. It was observed in a systematic investigation of the stages of peptic hydrolysis (2), that a slow, secondary hydrolysis of both proteose and peptone sets in after the first very rapid disintegration of albumin by pepsin.

An attempt was made to ascertain, regardless of the mechanism by which this effect is produced, whether the progressively lessening synthesizeability is due to the action of the pepsin, to the action of the acid, or to their combined action.

A large quantity of a peptic digest of egg albumin at pH 1.6 was prepared After a short interval a fraction was removed and boiled for one half hour in order to destroy the pepsin. Half was then concentrated at pH 4.0; the remainder was allowed to stand at room temperature with its hydrogen ion concentration at pH 1.6, which was the same as that of the main digest, until the last sample was removed from the main digest 23 days later. The reaction was then adjusted to pH 4.0 and this residual fraction was concentrated to the same extent as the first portion which had been prepared for synthesis immediately on removal from the main digest. In this manner a number of fractions were obtained which had been exposed to the combined action of the pepsin and a hydrogen ion concentration of pH 1.6 for varying lengths of time, and a second series in which each member had been exposed to the action of pepsin for the same periods as corresponding members in the first series, but in which the exposure to acid was the same for all as for that of the final sample which had suffered the action of the acid longest, *i.e.* 26 days. All the fractions were concentrated to the same nitrogen content. To 10 cc. of each fraction 1 cc. of a 10 per cent pepsin solution was added, and the mixtures were then set away at 37°C. for two weeks, a period which is adequate for the attainment of equilibrium.

It was found, as before, that the yield of protein synthesized progressively diminished with the length of time which the digest had been exposed to the pepsin; and further that exposure to a hydrogen ion concentration of pH 1.6 in the absence of active pepsin did not affect the amount of the yield. The fractions, in which the reaction was maintained at pH 1.6 for 23 days, 16 days and 9 days after destruction of the pepsin, gave the same yields of plastein as the corresponding fractions which were adjusted to pH 4.0 and concentrated immediately after removal from the main digest. These results indicate that the diminution in synthesizeability of a digest on exposure to pepsin at pH 1.6, is due to the action of the pepsin, and not of the acid, and is presumably a secondary hydrolysis.

It seems improbable that the specificity, *i.e.* the complex essential for peptic synthesis, resides in the amide linkage, in spite of the fact that the product of peptic synthesis contains fewer free amino and free carboxyl groups than any of the constituents of the digest. Were this

specificity associated only with the amide linkage, it would be difficult to account for the failure to effect synthesis in a digest of gelatine, or for the small yields obtainable from a gliadin digest. The problem is the same as in the enzymatic hydrolysis of proteins. Pepsin, trypsin, and erepsin all increase the number of the free amino and free carboxyl groups, yet show a distinct and characteristic specificity in the substrates susceptible to their action.

This effect of the duration of hydrolysis on the yield of protein synthesized precludes, it seems, the possibility that the essential radicle is a single amino acid, or a dipeptide. The latter substances are, as far as is known, not changed by pepsin, and are not, therefore, affected by any length of exposure to the action of that enzyme. Furthermore, the addition of the deficient amino acids to a digest of gelatine, does not remove the inability of such a solution to support peptic synthesis.

It was found that synthesis could also be effected in concentrated solutions of isolated fractions of a peptic digest, *i.e.* of proteose and of peptone. The yields were approximately the same as in similar concentrations of the whole digest, though the proteins so synthesized differed in some respects from those obtained from the whole digest. Assuming the existence of the hypothetical complex suggested above, the synthesizeability of both isolated proteose and peptone would indicate its wide distribution in the protein molecule (or at any rate among its cleavage products).

A series of experiments were carried out which constituted, in a sense, the converse of those, discussed above, in which the synthesizeability of digests hydrolyzed for different periods was measured.

Pepsin was added to a concentrated peptic digest and synthesis was permitted to proceed until no more protein was synthesized. The synthesized protein was removed by filtration after dilution, the filtrate was reconcentrated, its reaction readjusted to pH 4.0 and synthesis again induced with pepsin. This was repeated three times, so that four crops of protein were obtained from one original portion of concentrated digest.

In each case the reaction was allowed to proceed long enough to ensure a maximum yield. The four yields diminished progressively. The cessation of synthesis in any one digest obviously was not due to complete utilization of all the material essential for synthesis. The sim-

plest explanation is that the cessation in each case was due to the attainment of true equilibrium. A previous experiment had led to a similar conclusion. There, artificially synthesized insoluble protein, added to a concentrated solution of digest and active pepsin, was found to inhibit subsequent synthesis to a degree directly proportional to the amount added (3). The result seemed anomalous at that time in that the added protein, a substance apparently not in solution, had influenced the equilibrium position just as if it had been in solution. A similar phenomenon was observed later in the inhibition of hydrolysis by coagulated egg albumin (4).

Recent observations indicate that the synthesized protein is probably soluble to a considerable extent in the concentrated digest, though it is thrown completely out of solution on dilution. When a concentrated digest containing pepsin was poured into a wide, flat dish and left uncovered, the suspension, opaque and creamy in colour on account of insoluble protein, with the evaporation of water, became presently clear and transparent. On the addition of water the opacity reappeared. The same phenomenon was observed during the first few minutes of synthesis in a concentrated solution of proteose. The mixture remained quite clear, yet the occurrence of synthesis was demonstrable by the addition of trichloroacetic acid, by heating quickly to boiling, or by the addition of water. The latter observations on solutions of proteose were suggested by similar observations of Sawjalow (5).

EXPERIMENTAL

The digests employed were peptic digests of egg albumin. Dried egg white (Merck) was dissolved in N/10 HCl and digested with pepsin (Merck) at pH 1.6 and 37°C. for the lengths of time indicated. The hydrolysis was stopped by heating the digest in boiling water for ½ hour. Its reaction was then adjusted to pH 4.0, after which it was filtered and concentrated on a water bath.

Synthesis was carried out as described below for individual experiments. Measured amounts of a solution of pepsin were added to a known volume of digest. The solutions were thoroughly mixed by violent shaking and then incubated at 37°C. Chloroform was added as preservative. At the end of the period indicated the digest was diluted approximately twenty times. The extent of synthesis was estimated by the determination of the total nitrogen before and after precipitation by trichloroacetic acid.

The following experiment shows the progressive diminution in the yield of synthesized protein with the length of time exposed to the hydrolyzing action of the enzyme.

Ten litres of a 5 per cent solution of egg white were digested with pepsin. From time to time 2000 cc. portions were removed and prepared for synthesis as described above. Various lengths of time were allowed to elapse before the destruction of the enzyme and all the digests were concentrated to approximately the same nitrogen content. Two different enzyme concentrations were employed for synthesis. The results in each case were essentially the same as those given in Table I.

TABLE I

The Effect of Duration of Hydrolysis on the Subsequent Yield of Protein Synthesized by Pepsin

Duration of hydrolysis	Duration of synthesis	Total N in 5 cc. conc. digest	N of filtrate in 5 cc. conc. digest	N of synthesized protein in 5 cc. conc. digest
days	*days*	*mg.*	*mg.*	*mg.*
6	3	660	482	178
	11	662	464	198
	15	658	462	196
13	3	682	560	122
	11	674	522	152
	15	676	526	150
21	3	636	548	88
	11	654	522	132
	15	660	524	136
40	3	690	616	74
	11	684	596	88
	15	688	598	90

In order to ascertain whether the lessened synthesizeability on long digestion was due to the action of the pepsin, or of the acid, or of their combined action, 8 litres of 4 per cent egg albumin were digested at 37°C. with 0.4 per cent pepsin at pH 1.6. After 3 days the clear supernatant digest gave no precipitate with 2 per cent trichloroacetic acid. 2 litres were removed and boiled for ½ hour. One litre was then set away at room temperature; the remainder was filtered from the slight flocculant precipitate produced by boiling, and concentrated, after adjustment of its reaction to pH 4.0, so that 550 mg. nitrogen were contained in 10 cc. of solution. A similar procedure was repeated at intervals of 10 days and 17

days after the beginning of digestion. After 26 days the remainder of the digest was boiled and filtered, its reaction adjusted to pH 4.0, and concentrated. The three 1 litre portions in which the pepsin had been previously destroyed at various times, and which had been standing at room temperature since then, were now filtered, their hydrogen ion concentrations adjusted to pH 4.0, and concentrated on water baths.

The final concentrations of the fractions were, as nearly as they could be made, all the same. 1 cc. of 10 per cent pepsin, and 1 cc. of chloroform were added to 10 cc. of each of the digests, the containing vessels were stoppered and set away at 37°C. for 2 weeks. The contents were then analyzed for plastein. The results, given in Table II, show the diminution with prolongation of exposure to active pepsin of the amounts of protein synthesized, and the absence of any significant effect of acid alone.

TABLE II

The Effect of Exposure to Pepsin and to Acid on the Synthesizeability of a Peptic Digest of Egg Albumin

Duration of exposure to pepsin at pH 1.6	Subsequent exposure to a C_H of $10^{-1.4}$	Nitrogen content of 10 cc. of concentrated digest	Synthesis
days	*days*	*mg.*	*per cent of total nitrogen*
3	—	550	42
3	23	570	40
10	—	540	30
10	16	560	29
17	—	550	25
17	9	570	25
26	—	550	20

It had often been observed previously that the highest yields of plastein were obtained when the pepsin was destroyed as soon as all the dissolved protein was digested. The duration of hydrolysis in these cases was not more than 2 days. Under conditions similar to those of Table I the yield was regularly between 230 to 260 mg. N, in 5 cc. of concentrated digest.

The lessened synthesizeability of the digests hydrolyzed for a longer period cannot, it seems, be due to any internal anhydride formation. The ratio of free amino N to total N rose from 23 per cent in the 6 day digest to 27 per cent in the 40 day digest. Internal anhydride formation would have been accompanied by a reduction in this value. The

more rational interpretation is that the radicle essential for synthesis, liberated in the rapid primary hydrolysis, is destroyed in the much slower secondary hydrolysis. The disappearance of this essential compound during the secondary hydrolysis may be taken as evidence that it is more complex than a single amino acid. Since dipeptides are hydrolyzed only by erepsin, it may be assumed that the compound is more complex than a dipeptide.

These results suggest an explanation for the varying synthesizeability of peptic digests of different proteins. Albumin and globulin digests give high yields. Gliadin on the other hand gives low yields and gelatine digests have so far given consistently negative results. The results with the last two proteins can be interpreted in the light of the above results as due either to the failure of appearance of the essential complex hypothecated above during the primary hydrolysis, or to its more rapid destruction. This suggested explanation is in accordance with the variation in internal chemical structure of proteins, which is indicated by the variation in their rates of hydrolysis by enzymes.

Henriques and Gjaldbäk (8) observed that when proteins are partially digested by weak acid or alkali the concentrated digests yield little or no plastein on the addition of pepsin. This observation affords some ground for the belief that the complex essential for peptic synthesis is destroyed by strong acid or alkali, and may help to explain the non-synthesizeability of digests of gelatine because this protein is prepared from collagen by treatment with strong acid.

For the preparation of the proteose and subproteose fractions employed in the experiments discussed above, 9 litres of a peptic digest of albumin were employed. It was boiled for ½ hour and after cooling 360 cc. 10 per cent NaOH were added to bring the reaction to pH 4.0. It was then filtered and from the clear filtrate the proteose was salted out with anhydrous Na_2SO_4 at 33°–36°C. The proteose rose to the surface in a thick, sticky mass. It was skimmed off, redissolved in distilled water and reprecipitated with the same procedure three times. It was found that very little salt had adhered to the proteose, so that no procedures were necessary to remove it as was the case with the subproteose fraction.

The solution from which the proteose was removed was filtered while still warm in order to free it from suspended particles of salted out proteose. The solution was then cooled to about 7°C. to precipitate as much of the hydrated salt as possible. As the anhydrous salt was added and the hydrated form precipitated, a considerable concentration of the solution was attained. To the cold filtrate from

the precipitated salt enough methylated alcohol was added to bring the concentration to 50 per cent. Practically all the salt and none of the nitrogenous constituents are precipitated in this concentration of alcohol. The alcohol was evaporated *in vacuo* at 40°C.

Both the proteose and subproteose fractions, dissolved in water, and free of salt, were concentrated over a water bath to the same nitrogen concentration; they contained 3000 mg. of nitrogen in 100 cc., corresponding to approximately 20 per cent of material.

To 200 cc. of each of these solutions 4 gm. pepsin were added. The peptone solution very soon became opaque. The proteose, however, remained clear for a much longer time, becoming opaque only after a number of hours. This was due not, as it proved, to a slower rate of synthesis in the proteose solution, but to the fact that the protein synthesized in the proteose digest is soluble in the concentrated solution of proteose.

The synthesized protein is slowly denatured, being converted to an insoluble form on standing in the concentrated digest at room temperature. The rate of this denaturation is greater at temperatures above 30°C. It was observed that the protein synthesized from proteose alone was distinctly less soluble in a dilute solution of products than in a more concentrated one. A heavy precipitate of protein is obtained from the concentrated digest by 2 per cent trichloroacetic acid, whereas addition of an equal volume of water induces only a turbidity. If the concentrated solution of proteose containing the protein synthesized from it is brought quickly to the boil, the protein is precipitated as a coagulum.

In the peptone solution the protein appeared to be precipitated as soon as it was formed.

The yields obtained corresponded to those found with similar concentrations of a complete digest. With a concentration of 20 per cent of material in solution the following yields were obtained. Proteose alone yielded 26 per cent, peptone alone 17 per cent, and a mixture of equal parts of similar concentrations of proteose and peptone 22 per cent.

In order to examine the product of synthesis more closely, more proteose and peptone were prepared. Their solutions were concentrated and proteins synthesized from them, with pepsin, as described. The proteins were isolated by dilution of the suspension of synthesized protein and digest to 10 times the volume with distilled water. The precipitated proteins were washed thoroughly, first by

decantation and finally on a filter paper until the wash water gave a negative reaction for chlorides. The proteins were then washed with absolute alcohol and air dried. The free COOH and amino nitrogen in the protein prepared from proteose was measured and also the rate of its hydrolyzeability, and the results compared with those obtained on the substrate from which it was prepared. The free COOH groups were determined by the method of Willstätter (6), and the free amino nitrogen by the micro method of Van Slyke. The results are given in Table III.

TABLE III

A Comparison of the Chemical Characteristics of Proteose with Those of Protein Synthesized from It

	Total N in 10 cc.	Equivalents × 14 of COOH groups in 10 cc. soln.	$\frac{\text{COOH equiv.} \times 14}{\text{Total N}}$	Free amino N in 10 cc. solution	$\frac{\text{Free amino N}}{\text{Total N}}$
	mg.		*per cent*	*mg.*	*per cent*
Proteose	15.3	3.8	24.9	2.01	13.2
Protein synthesized from proteose	9.2	1.19	12.9	0.99	10.8

TABLE IV

The Action of Acid on Proteins Synthesized from Proteose and Peptone

	Proteose protein		Peptone protein	
	Before soln. in acid	After soln. in acid	Before soln. in acid	After soln. in acid
	mg. N	*mg. N*	*mg. N*	*mg. N*
Free COOH as N/5 in 5 cc.	0.5	1.2	0.5	1.2
Free amino N, mg. in 2 cc.	0.198	0.409	0.39	0.52

Only the rate of its hydrolysis by pepsin could be measured in the protein synthesized from peptone owing to the small amount of material available.

The protein synthesized from proteose is hydrolyzed much more slowly than the plastein synthesized from the whole digest, and similar results were obtained with the protein synthesized from peptone alone.

When dissolved in dilute acid, so that the pH of the solution is in the neighborhood of 1.6 the proteins synthesized from both proteose and

from peptone undergo certain changes which result in the opening up of a considerable number of amide linkages. Large increases in the free amino N and in the free COOH groups occur. These are set out in Table IV.

An analogous phenomenon has been observed by Cohn and Berggren (7) in the effect of alkali on casein. In this case however the increase in COOH groups was exactly equivalent to the increase in NH_2 groups.

Synthesizeability of Residual Digest after Synthesis

300 cc. of a concentrated peptic digest (35 per cent) were set away with 60 cc. of 15 per cent pepsin and 1 cc. of $CHCL_3$ at 37°C. The concentration of pepsin was made so high in order to obtain as large a yield as possible. At the end of 10 days the digest was diluted to 2.5 litres. 3–5 cc. portions were taken for total nitrogen. 40 cc. were pipetted into 10 cc. of 10 per cent trichloroacetic acid and the total nitrogen of the filtrate was determined. The two Kjeldahl determinations gave, after correction for dilution, the amount of protein synthesized. The remainder of the diluted digest was filtered so that all but a trace of the synthesized protein was removed. The filtrate was reconcentrated, after adjustment of the reaction to pH 4.0, until the amount of digest nitrogen, in contradistinction to nitrogen contained in the pepsin added, was the same as in the original concentrated digest before the first synthesis had occurred. More pepsin was added, and the mixture was incubated again at 37°C. At the end of 10 days the digest, now again containing synthesized protein, was again diluted and the procedure described above repeated. This was repeated three times, so that four yields of protein were synthesized from the one digest.

The details are as follows:—

300 cc. digest + 60 cc. 15 per cent pepsin + 1 cc. $CHCl_3$ incubated for 10 days at 37°C. The digest contained 8700 mg. nitrogen in 100 cc. exclusive of nitrogen added in the pepsin. Of this nitrogen 36.3 per cent was synthesized to protein and therefore removed from the solution.

The filtrate from the 2.5 litres of diluted digest was concentrated to 230 cc., 46 cc. of 15 per cent pepsin and 1 cc. chloroform were added, and the digest was again set away at 37°C. The concentration of digest nitrogen was 6400 mg. in 100 cc. although the actual nitrogen content after addition of pepsin was 6540 mg. per 100 cc. After 10 days incubation the digest was diluted to 1850 cc., 3–5 cc. were taken for total N and 40 cc. for precipitation with trichloroacetic acid and determination of the total nitrogen on the filtrate as before. The per cent synthesis here was 11 per cent.

1850 cc. of the second filtrate were concentrated to 170 cc., 40 cc. of 15 per cent pepsin and 1 cc. $CHCl_3$ were added. It was set away again at 37°C. for 10 days. The concentration of digest nitrogen was 6400 mg. per 100 cc. At the end of the 10 day period it was diluted to 1900 cc. The per cent synthesis here was 4 per cent.

1880 cc. were concentrated to 160 cc. 37.5 cc. of 15 per cent pepsin and 1 cc. chloroform were added, and it was set away at 37°C. for 10 days. The per cent synthesis in this 4th crop was 0.7 per cent.

These results show that the cessation of synthesis in any one digest cannot be entirely due to complete utilization of a material essential for synthesis. If that had been the case no synthesis could have been obtained from the first filtrate. The pepsin added does not supply any synthesizeable material. In spite of the fact that commercial pepsin contains proteoses and peptones no synthesis can be obtained from its concentrated solution. Moreover, the very low yield from the third filtrate where there was a considerable amount of pepsin nitrogen, indicates that the enzyme preparation could not have been the source of the synthesizeable material in the first and second filtrates.

SUMMARY

1. Experiments are described in which it was observed that the yield of protein that can be synthesized by pepsin from a given peptic digest is highest when the hydrolyzing action of the pepsin is stopped as soon as all the protein has disappeared from the solution; and that the longer the digest is permitted to contain active enzyme the more the yield diminishes.

2. Exposure of the digest to a hydrogen ion concentration of pH 1.6 in the absence of active enzyme, does not cause a diminution in the amount of protein which can be synthesized from that digest.

3. Synthesis can be effected also in concentrated solutions of isolated fractions of a peptic digest, *i.e.* of proteose and of peptone. The yields are approximately the same as in similar concentrations of the whole digest, though the proteins so synthesized differ in some respects from those obtained from the whole digest.

4. The cessation of synthesis in any one digest is due to the attainment of equilibrium and not to the complete utilization of available synthesizeable material. The amount of the equilibrium yield, on the

other hand, is dependent on the amount of synthesizeable material in the digest.

5. These observations are taken to show that the synthesizeability of a given mixture of protein cleavage products by pepsin depends upon its possession of a special complex in these products. This complex appears as a result of the primary hydrolysis of the protein molecule by pepsin and is decomposed in the slow secondary hydrolysis which ensues as digestion is prolonged.

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A CRYSTALLOGRAPHIC STUDY OF PURE CARBONMONOXIDE HEMOGLOBIN

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PLATES 3 TO 5

(Accepted for publication, October 31, 1929)

In their treatise on the crystallography of hemoglobin Reichert and Brown[1] recorded literature representing the history of hemoglobin crystallization to the date of its publication, 1909. Hünefeld[2] was given credit for being the discoverer of crystals of hemoglobin in 1840. K. E. Reichert[3] might be called the true discoverer of the oxyhemoglobin crystals, however, for his discovery is the actual foundation of our knowledge of hemoglobin. Six hours after the death of a guinea pig he found crystals on the maternal uterus and in the placentas of all four fetuses present. Funke[4] was said to be the first to prepare "blood crystals," 1851. They quoted Preyer's[5] monograph, "Die Blutkrystalle," as "the leading authority on the crystallography of hemoglobin" up to that time. Preyer[6] classified some biological species he studied according to the crystal system of the oxyhemoglobin crystals. But by far the most comprehensive monograph on the subject published up to the present time is that of Reichert and

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Brown.[1] This memoir on the crystallography of hemoglobin refers particularly to the relationships occurring in its biological and crystallographical classifications.

They concluded that genus and species specificity are indicated by hemoglobin crystal structure. However, improvements may be made in the method of crystal preparation. In no case reported in that treatise did the investigators use pure compounds in the crystal preparations. Most of the crystals were prepared for microscopical observation as follows:

> The blood, if available in small quantity only, was dropped on a microscopic slide, laked with a drop or two of water or ether, ammonium sulfate or oxalate added, and the material allowed to concentrate by evaporation. Then it was covered with a cover-glass, and this sealed with balsam to insure only slow evaporation. Crystals appeared when kept in the refrigerator for from a few hours to months. If the material was available in sufficiently large quantities, the red corpuscles were settled from the oxalated or defibrinated blood by centrifuging, laked with ether, saturated with ammonium oxalate, centrifugalized, and crystallized on a microscopic slide, with the cover glass sealed with balsam. When crystallization was too rapid a retardant was used, such as plasma, egg white, etc. When crystallization did not take place at a satisfactory speed, the workers used reduced hemoglobin or carbonmonoxide hemoglobin. Methemoglobin and metoxyhemoglobin were crystallized in some cases. Blood which had stood for several days was sometimes used, often being clotted and even putrified.

It is suggested that the various organic impurities might have influenced the crystal habit, and even the crystal system and form. There is no evidence indicating that hemoglobin and plasma proteins or egg white proteins or stroma proteins do not form chemical combinations. If such a combination were present the crystals were those of this complex material, rather than of the pure hemoglobin derivative, as the authors quoted indicated. Crystal shape may have been influenced by occluded proteins from stroma and serum. When carbonmonoxide hemoglobin was desired the blood or corpuscular paste was saturated with illuminating gas. Of course this was not pure carbonmonoxide, and it is possible that derivatives other than carbonmonoxide hemoglobin were produced.

The oxyhemoglobins and carbonmonoxide hemoglobins, crystal photographs of which are appended, were carefully prepared by the

method described elsewhere.[7] This method was essentially as follows:

Oxyhemoglobin was prepared by the method of Marshall and Welker.[8] Red corpuscles were separated by centrifuging, washed with saline solution, and laked with water. The solution was mixed with aluminum cream and filtered in a refrigerator. The filtrate contained no protein other than oxyhemoglobin as was shown by Marshall and Welker in 1913. The aluminum cream was prepared by adding slowly, with constant stirring, a 1 per cent solution of ammonium hydroxide to a 1 per cent solution of ammonium aluminum sulfate until the reaction was slightly alkaline. The precipitate formed was repeatedly washed by decantation until the supernatant liquid gave no test for ammonia with Nessler reagent. Carbonmonoxide hemoglobin was prepared by saturating oxyhemoglobin with CO gas generated by warm formic acid and concentrated H_2SO_4. The gas was twice washed with NaOH and H_2O. The oxyhemoglobins and carbonmonoxide hemoglobins were recrystallized from absolute alcohol distilled from lime, at 0°C. or below. The amount of alcohol required for crystallization was 5 per cent or less in the case of the rat oxyhemoglobin and carbonmonoxide hemoglobin, increasing with guinea pig, horse, dog, turkey, chicken, approximately in this order to 38 per cent for ox, sheep and hog carbonmonoxide hemoglobin.

These oxyhemoglobins and carbonmonoxide hemoglobins were presumably pure chemical compounds, although in a few cases crystals of two varieties may have formed from the same solution, *e.g.*, chicken carbonmonoxide hemoglobin, dog oxyhemoglobin and hog carbonmonoxide hemoglobin. In several preparations, as horse oxyhemoglobin and carbonmonoxide hemoglobin, and dog and rat oxyhemoglobin, Reichert and Brown found varieties of crystals which they designated as α and β hemoglobin or carbonmonoxide hemoglobin. Bohr[9] had previously claimed that more than one kind of hemoglobin may occur in the same animal. In the work now being reported only one kind of crystal of horse oxyhemogobin and of horse carbonmonoxide hemoglobin appeared. Dog oxyhemoglobin may be represented by two forms, but only one form can be detected in dog carbonmonoxide hemoglobin. The brown rat β oxyhemoglobin, so-called by Reichert and Brown, may be according to them an isomer or

[7] Boor, Alden K., and Hektoen, Ludvig, *J. Inf. Dis.*, 1930, **46**, 1.
[8] Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, **35**, 820.
[9] Bohr, Christian, *Skandin. Arch. f. Physiol.*, 1892, **3**, 76.

mimetic twin of the α form. No β oxyhemoglobin crystal from rat was observed in this work. Only one crystal form of rat carbonmonoxide hemoglobin appeared. Reichert and Brown only report one chicken oxyhemoglobin, although they say that they "sometimes look tetragonal but are orthorhombic according to optical characters." Figs. 20 and 21 show two kinds of crystals of chicken carbonmonoxide hemoglobin. Whether these belong to different systems is a question. Optical character was not investigated, but it is possible that the observation of Reichert and Brown holds also for the crystals of Figs. 20 and 21. Every case of dual form is questionable: the crystals investigated in this work suggest one hemoglobin per species.

Photomicrographs of crystals of carbonmonoxide hemoglobin of ox, sheep, hog, dog, turkey, rat, horse, chicken and guinea pig, and oxyhemoglobin of horse, hog, ox, sheep, dog, guinea pig and rat are shown. The photomicrographs were taken outdoors, in winter, when the temperature was below 0°C., using a Zeiss photomicroscope. No attempt was made to make a thorough study of the crystals at the time of photographing. All observations and conclusions were based on a study of the photographs.

Ox

Reichert and Brown described their ox oxyhemoglobin crystals essentially as follows: prismatic, belonging to the orthorhombic system and about four times as long as the width. Twinning was reported.

In Figs. 1 and 2 there seems to be one form, evidently long, lath-shaped, prismatic crystals, probably belonging to the orthorhombic system. Much longer, thinner crystals were obtained than those described by Reichert and Brown. Twinning is also observed.

Figs. 3 and 4 represent ox carbonmonoxide hemoglobin crystals which seem to be orthorhombic and appear much like the bison oxyhemoglobin crystals described by Reichert and Brown as orthorhombic. The brachydome angle of 125° measured by them is the same as angle of brachydome of bullock or ox oxyhemoglobin crystals observed by them.

Sheep

The preparation of sheep oxyhemoglobin was crystallized by Reichert and Brown at room temperature, some crystals being obtained within 5 hours of making the preparation. That represented by appended photographs was rather difficult to crystallize. Reichert and Brown report that needles, without definite outline, tapering to a point at either end, formed at first, but soon tabular crystals consisting of the base with a very short prism began to appear. Then after about a day, long, prismatic crystals appeared consisting of three pinacoids, elongated parallel to the vertical axis and generally flattened on the orthopinacoid.

On Figs. 5 and 6 no needles and no tabular crystals of type noted by Reichert and Brown are seen. All crystals are trapezoidal shaped: perhaps all are the "horse-type twins" of Reichert and Brown, these twins being united along a prism-base edge. Both penetrating and contact twins are illustrated.

The carbonmonoxide hemoglobin crystals of Figs. 7 and 8 are tabular entirely, being thin and fragile. They are orthorhombic in general although perhaps one shown is tetragonal.

Hog

Reichert and Brown report hog oxyhemoglobin crystals of the orthorhombic system, of short prismatic habit; twinning was not observed.

Figs. 9 and 10 indicate a thin, fragile, tabular form crystal with base almost square, and also, lathlike platelets about ten times as long as wide with rectangular ends. No crystals of the shape indicated by Reichert and Brown appear. Twins are present, being noticeable on some tabular form crystals.

On Figs. 11, 12, and 13 prismatic crystals of tabular form belonging to the monoclinic system appear. These are hog carbonmonoxide hemoglobin crystals. The axis perpendicular to the base is very short with respect to the other two. The four edges of the base are almost, if not quite equal. Twins of tufts and sheaf-like aggregates resemble Reichert and Brown's crystals of reduced hemoglobin.

Dog

Reichert and Brown's preparations of dog oxyhemoglobin were made as follows: it was prepared from whole blood "defibrinated by beating, and from blood kept liquid by oxalating, and also from mixtures of whole blood and blood plasma. The blood, either defibrinated or oxalated, was laked with ether and centrifugalized; and from the clear solution thus obtained, with or without the addition of plasma, the slide preparations were made." They found crystals of two types—orthorhombic which they called α oxyhemoglobin, and monoclinic which was designated as β oxyhemoglobin. Crystals of β oxyhemoglobin were only occasionally observed by them, "in perhaps one out of a dozen slides" and appeared to develop more readily in the blood to which no oxalate had been added.

In Figs. 14 and 15 are also found crystals which resemble the orthorhombic α oxyhemoglobin and the monoclinic β oxyhemoglobin of Reichert and Brown. The first type is evidently rectangular while the second has a base of rhombus shape which perhaps occur in trapezoidal "horse-type twins." As in the work cited above there are many more of the α oxyhemoglobin type than of the β type.

Figs. 16 and 17 show dog carbonmonoxide hemoglobin crystals of only one type. They are prismatic, orthorhombic and are generally shorter than the oxyhemoglobin, the ratio of length to the thickness varying from about 2:1 to 5:1.

Turkey

Turkey oxyhemoglobin crystals were not photographed.

Turkey carbonmonoxide hemoglobin crystals, Figs. 18 and 19, are probably of one variety, being tetragonal or orthorhombic, with the base edge about three times the vertical edge. Another form of crystal may be present, but this questionable figure is probably a plate on edge; the length is about three times the thickness.

Chicken

The chicken carbonmonoxide hemoglobin, Figs. 20 and 21, show crystals similar to those of Reichert and Brown found in the case of chicken oxyhemoglobin. They found orthorhombic crystals which "sometimes look tetragonal but are orthorhombic according to optic

characters." In both cases square tabular crystals occurred. Reichert and Brown's crystals aggregated into groups by piling up of the plates, or perhaps twinning on an axis normal to an edge (110–001); also by what appeared to be twinning on a dome, the crystals usually occurring in isolated clusters. On Figs. 20 and 21 some twinning on edge may be seen. The second kind of crystal is prismatic, with length about ten times the width.

Rat

Reichert and Brown examined oxyhemoglobin from four kinds of rats, white, brown, black and Alexandrine. Slight differences were found in the crystals from the Norway and albino rat on the one hand, and the Alexandrine and black rat on the other hand. In all four cases, however, orthorhombic crystals were found. These were prismatic, elongated, six-sided plates. A β oxyhemoglobin was found in the blood of the brown rat. It was isotropic and apparently isometric with the α oxyhemoglobin, but showed hexagonal outlines.

The material from which crystals of Figs. 22, 23, 24, and 25 were made was obtained from wild rats which, because of color variegations were thought to be crossed from white, black and Norway or brown varieties. Only one kind of oxyhemoglobin and one of carbonmonoxide hemoglobin was observed. Both appear to be elongated, hexagonal, tabular plates. The ratio of width to length is 1:5. They are both thin and fragile. The oxyhemoglobin in the accompanying photograph appears to be monoclinic.

The rat carbonmonoxide hemoglobin crystals, Figs. 24 and 25, are also elongated, hexagonal plates of tabular form. However, they probably belong to the orthorhombic system. They are about twice as long as the width. On Figs. 24 and 25 the original facial angles have been somewhat destroyed by exposure of the crystals to temperatures high enough to partially melt the edges.

Horse

Orthorhombic α oxyhemoglobin and α carbonmonoxide hemoglobin and β oxyhemoglobin and β carbonmonoxide hemoglobin crystals were obtained by Reichert and Brown from horse red corpuscles. Only one kind can be distinguished on photographs of pure horse

oxyhemoglobin (Figs. 26 and 27) and pure carbonmonoxide hemoglobin (Figs. 28, 29, and 30). The oxyhemoglobin may have the form of Reichert and Brown's oxyhemoglobin, appearing to be monoclinic prisms and all being fishtail, gypsum-type twins. The horse carbonmonoxide hemoglobin, on the other hand, seems to resemble the oxyhemoglobin in form, appearing to be tabular, orthorhombic crystals. Reichert and Brown report that long, hair-like needles form first which soon become more or less stout prisms. The length-width ratio of crystals vary from 8:1 to 50:1 for carbonmonoxide hemoglobin, and 10:1 in the case of oxyhemoglobin.

Guinea pig

Crystals of guinea-pig oxyhemoglobin and carbonmonoxide hemoglobin formed very easily. Orthorhombic, sphenoidal crystals appeared in both cases, as was reported by Reichert and Brown in the case of oxyhemoglobin. Most of the crystals on Photographs 31, 32, 33, and 34 seem to be right-handed sphenoid with corners truncated by left-handed sphenoid.

SUMMARY AND CONCLUSIONS

1. Photomicrographs of crystals of pure carbonmonoxide hemoglobin of the following species are presented; ox, sheep, hog, dog, turkey, rat, horse, chicken and guinea pig. Photomicrographs of the oxyhemoglobin crystals of the following species are also shown: ox, sheep, hog, dog, rat, horse and guinea pig. The crystals were formed from the pure protein by adding a suitable amount of ethyl alcohol and maintaining a temperature of 0°C., or lower.

2. In some species a sufficient difference is shown between the carbonmonoxide hemoglobin and oxyhemoglobin crystals to distinguish these compounds, but the photographs of crystals of carbonmonoxide hemoglobin and oxyhemoglobin of some species, such as guinea pig, show no appreciable difference.

3. Differences between the carbonmonoxide hemoglobins, as well as between the oxyhemoglobins, of the different species studied are indicated.

4. The carbonmonoxide hemoglobin crystals from the bloods studied are species specific in their nature, and, in many cases, can be dis-

tinguished from the analogous oxyhemoglobin by crystallographic study.

The author wishes to express his thanks and appreciation to Dr. William H. Welker, at whose suggestion this investigation was undertaken, for his kind advice, valuable assistance and inspiring encouragement in all of the work represented by this paper.

EXPLANATION OF PLATES

PLATE 3

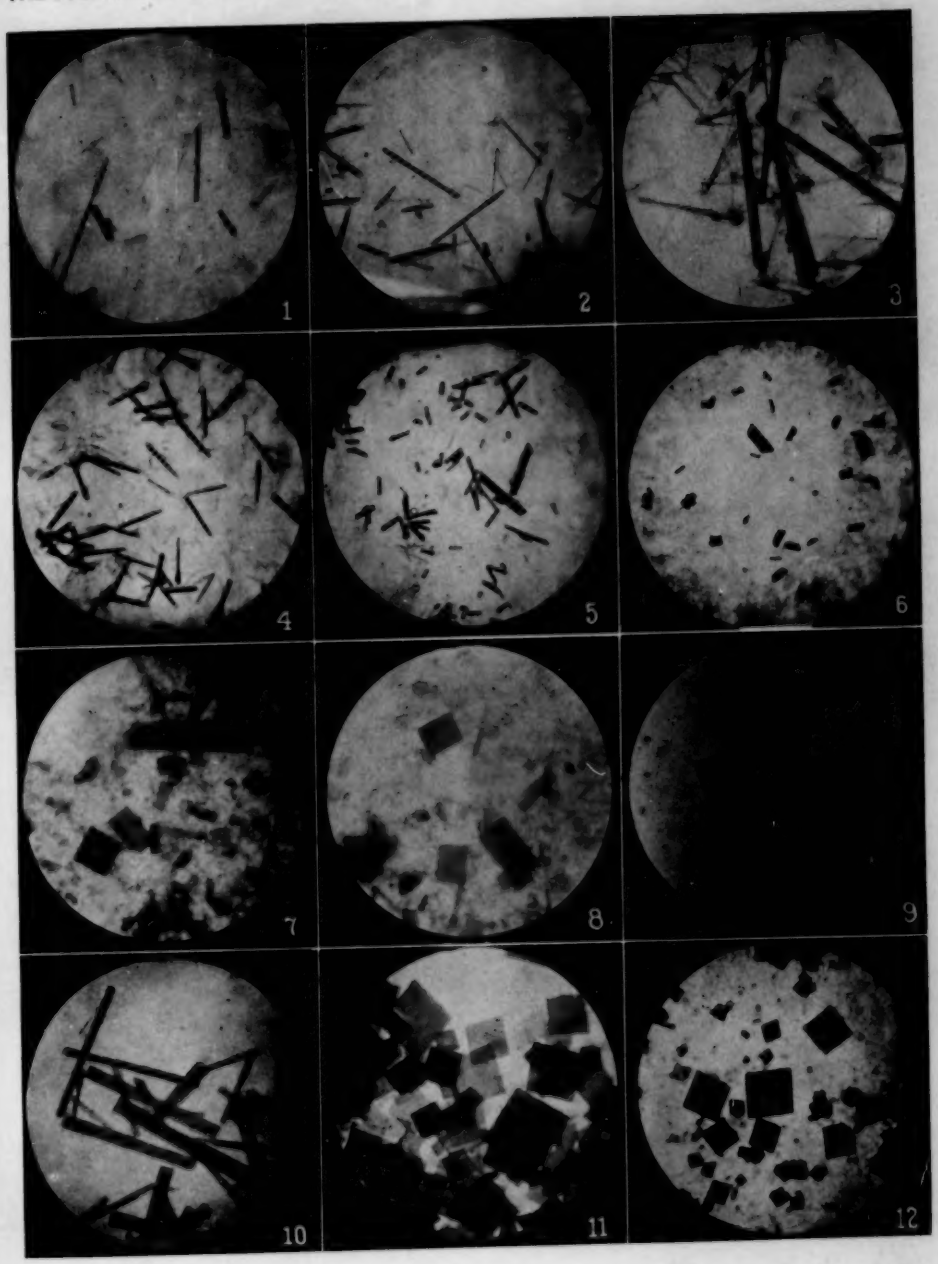
FIG. 1. Ox oxyhemoglobin. × 18.6.
FIG. 2. Ox oxyhemoglobin. × 18.6.
FIG. 3. Ox carbonmonoxide hemoglobin. × 62.
FIG. 4. Ox carbonmonoxide hemoglobin. × 18.6.
FIG. 5. Sheep oxyhemoglobin. × 18.6.
FIG. 6. Sheep oxyhemoglobin. × 18.6.
FIG. 7. Sheep carbonmonoxide hemoglobin. × 62.
FIG. 8. Sheep carbonmonoxide hemoglobin. × 62.
FIG. 9. Hog oxyhemoglobin. × 246.
FIG. 10. Hog oxyhemoglobin. × 62.
FIG. 11. Hog carbonmonoxide hemoglobin. × 62.
FIG. 12. Hog carbonmonoxide hemoglobin. × 62.

PLATE 4

FIG. 13. Hog carbonmonoxide hemoglobin. × 18.6.
FIG. 14. Dog oxyhemoglobin. × 14.1.
FIG. 15. Dog oxyhemoglobin. × 14.1.
FIG. 16. Dog carbonmonoxide hemoglobin. × 62.
FIG. 17. Dog carbonmonoxide hemoglobin. × 62.
FIG. 18. Turkey carbonmonoxide hemoglobin. × 14.1.
FIG. 19. Turkey carbonmonoxide hemoglobin. × 14.1.
FIG. 20. Chicken carbonmonoxide hemoglobin. × 62.
FIG. 21. Chicken carbonmonoxide hemoglobin. × 62.
FIG. 22. Rat oxyhemoglobin. × 62.
FIG. 23. Rat oxyhemoglobin. × 62.
FIG. 24. Rat carbonmonoxide hemoglobin. × 62.

PLATE 5

FIG. 25. Rat carbonmonoxide hemoglobin. × 62.
FIG. 26. Horse oxyhemoglobin. × 62.
FIG. 27. Horse oxyhemoglobin. × 62.
FIG. 28. Horse carbonmonoxide hemoglobin. × 14.1.
FIG. 29. Horse carbonmonoxide hemoglobin. × 188.
FIG. 30. Horse carbonmonoxide hemoglobin. × 62.
FIG. 31. Guinea pig oxyhemoglobin. × 62.
FIG. 32. Guinea pig oxyhemoglobin. × 62.
FIG. 33. Guinea pig carbonmonoxide hemoglobin. × 14.1.
FIG. 34. Guinea pig carbonmonoxide hemoglobin. × 18.6.



(Boor: Pure carbonmonoxide hemoglobin)

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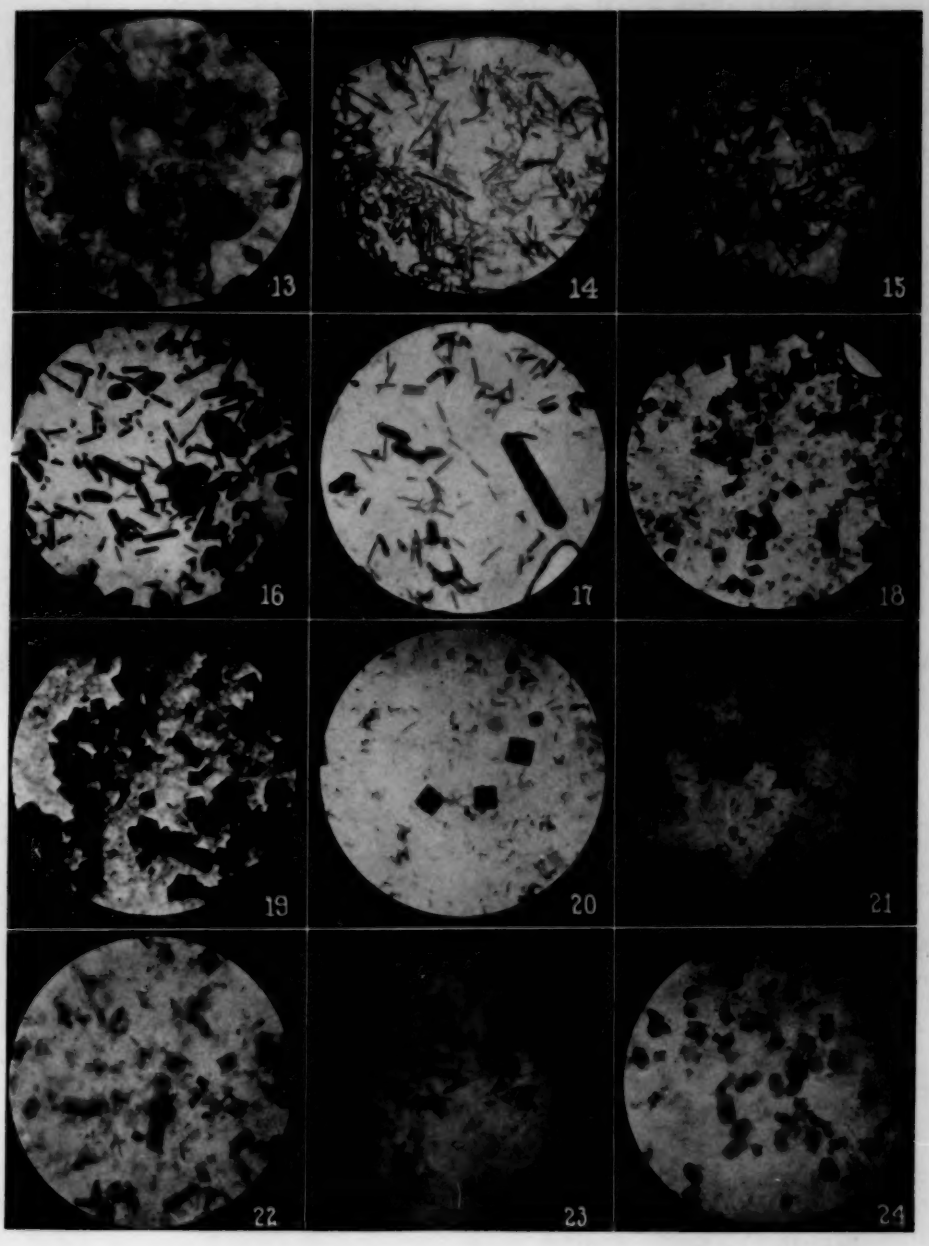

(Boor: Pure carbonmonoxide hemoglobin)

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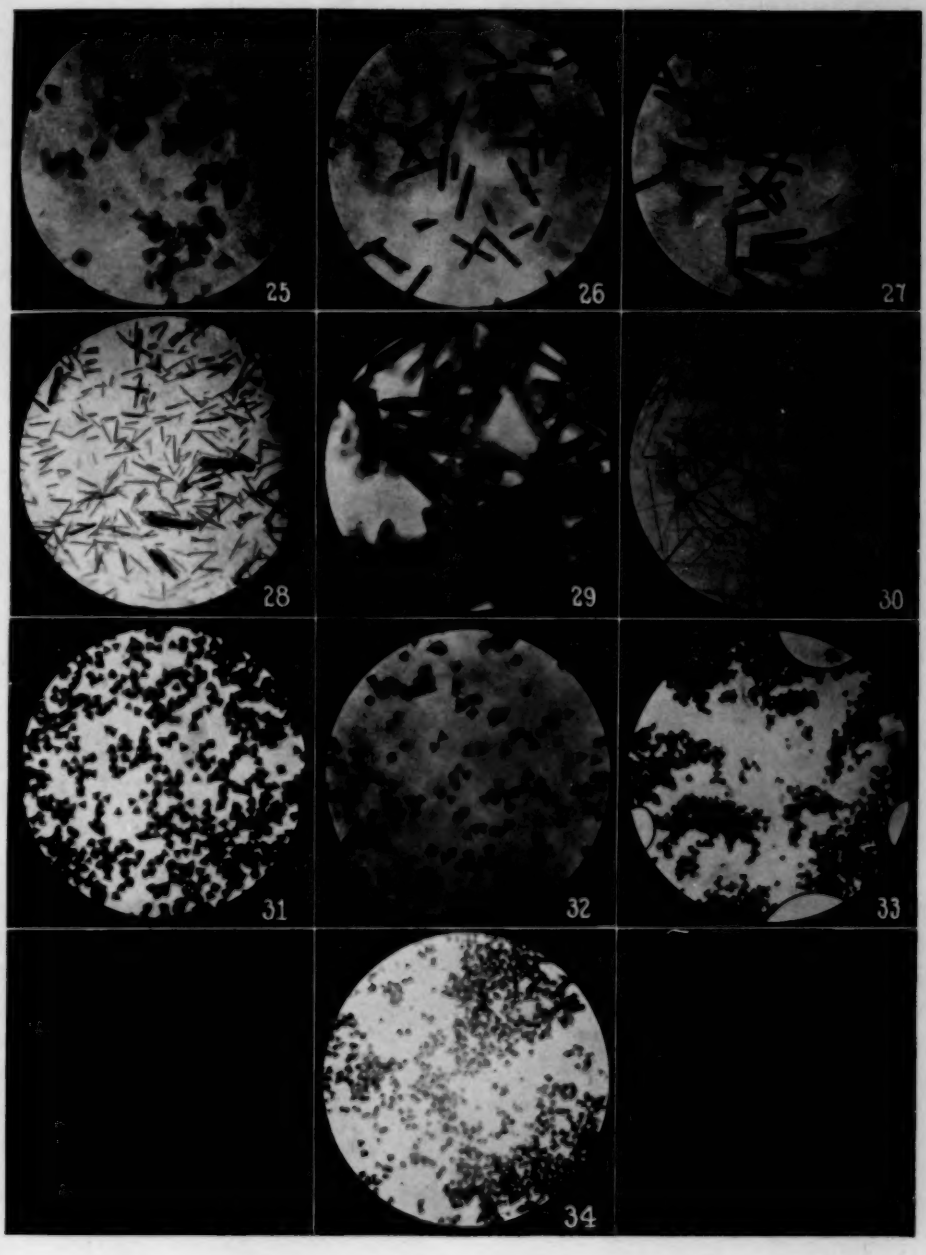

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NOTE ON THE VARIATION WITH TEMPERATURE OF THE RELATIVE RATES OF HYDROLYSIS OF GLUCOSIDES

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Comparisons of the rates at which two or more glucosides* undergo hydrolysis under similar catalytic conditions have been made by numerous workers, [1–8] the numerical value of the ratio of two unimolecular velocity coefficients being subsequently employed as evidence in support of the mechanism suggested for the hydrolysis. Thus, Armstrong and Caldwell,[3] from their observation that β-methylglucoside is hydrolysed 1.79 times as rapidly as α-methylglucoside at 74.1°C., argued that hydrolytic attack during acidic hydrolysis is concerned with the oxygen atom of the ring structure in the sugar portion of the molecule. Similarly, Kuhn and Sobotka,[6] employing the data of Sigmond,[2] Armstrong[3] and Fischer[5] on the rates of hydrolysis of maltose and α-methylglucoside at 74.1°C., arrive at the value of 0.14 for the ratio $\frac{k_{\alpha\text{-methylglucoside}}}{k_{\text{maltose}}}$; a comparison of this value with a similar ratio found for the enzymic hydrolysis of these glucosides at 30°C. is regarded by Kuhn and Sobotka as quantitative

* The term *glucoside* is here used in its general sense, *i.e.* to denote any compound one of the products of hydrolysis of which is a sugar.

[1] Ekenstein, *Rec. trav. Pays-Bas*, 1895, **13**, 185.

[2] Sigmond, *Z. physikal. Chem.*, 1898, **27**, 385.

[3] Armstrong and Caldwell, *Proc. Roy. Soc. London, Series B*, 1904, **74**, 188.

[4] Armstrong and Glover, *Proc. Roy. Soc. London, Series B*, 1908, **80**, 312.

[5] Fischer, *Z. physiol. Chem.*, 1919, **107**, 176.

[6] Kuhn and Sobotka, *Z. physikal. Chem.*, 1924, **109**, 65.

[7] Josephson, *Z. physiol. Chem.*, 1925, **147**, 1.

[8] Freudenberg, Dürr, and Hochstetter, *Berichte*, 1928, **61**, 1735.

support for the validity of the Euler-Michaelis[9] theory of catalysis in solution. It is noteworthy that, in almost all those cases where relative rates of hydrolysis are considered, the ratio of the velocity coefficients for the two glucosides which are compared at the same temperature is assumed to be independent of temperature. The present note is intended to demonstrate that this is true only in certain special cases, and that several of the disparities in values recorded for relative rates of hydrolysis may be attributed to the omission of this fact.

It is well known that all glucosides are hydrolysed by acids according to the unimolecular law;—

$$k = \frac{1}{t} ln \frac{A}{A - x} \quad \ldots\ldots (1),$$

and that the variation of k with temperature is given by the Arrhenius[10] equation;—

$$\frac{d \ ln \ k}{dT} = \frac{E}{RT^2} \quad \ldots\ldots (2).$$

Experiment shows that E, the critical increment or energy of activation, is, for the hydrolysis of glucosides by acids, independent of temperature, the degree of hydration, and the thermodynamic activity of the catalysing hydrogen ion.[11] In order to show how the *ratio* of the velocity coefficients for two glucosides varies with temperature, the case of the isomeric methylglucosides may be considered. Let k_{α_1} and k_{β_1} be the unimolecular constants for the hydrolysis of α-methylglucoside and β-methylglucoside respectively at a temperature T_1. If k_{α_2} and k_{β_2} be the corresponding values of the rates of hydrolysis at a temperature T_2, then it follows from equation (2) that;—

$$\frac{k_{\alpha_2}}{k_{\beta_2}} = \frac{k_{\alpha_1}}{k_{\beta_1}} e^{\frac{(E_\alpha - E_\beta)(T_2 - T_1)}{RT_2T_1}} \quad \ldots\ldots (3)$$

It is at once clear from this equation that the ratio of the rates of hydrolysis for two glucosides will be independent of temperature only

[9] Euler, *Z. physikal. Chem.*, 1900, **32**, 348; Michaelis, *Berichte*, 1913, **46**, 3683; Euler, *Trans. Faraday Soc.*, 1928, **24**, 651.

[10] Arrhenius, *Z. physikal. Chem.*, 1889, **4**, 226.

[11] Moelwyn-Hughes, *Trans. Faraday Soc.*, 1929, **25**, 81.

when the two hydrolyses have identical critical increment values. It has been shown[12] that E for the hydrolysis of α-methylglucoside is greater than E for the hydrolysis of the β isomer, hence the ratio $\frac{k_\alpha}{k_\beta}$ increases with rise in temperature.[13] At a temperature of 116°C. this ratio becomes unity, *i.e.* both glucosides are hydrolysed at the same rate; above this temperature, α-methylglucoside is the more

Glucoside hydrolysed	Critical Increment (Calories/gram mole)
Disaccharide	
Trehalose	40,180
Melibiose	38,590
Gentiobiose	33,390
Turanose	32,450
Maltose	30,970
Cellobiose	30,710
Lactose	26,900
Sucrose	25,830
Trisaccharides	
Melezitose	25,600
Raffinose	25,340
Glucosides	
α-Methylglucoside	38,190
Mandelonitrile-glucoside	34,040
β-Methylglucoside	33,730
Salicin	31,630
Arbutin	30,760
Phlorhizin	22,920
Tetramethyl-α-methylglucoside	19,840

readily hydrolysable; below this temperature, β-methylglucoside is the more easily attacked. Since the discovery of the isomeric methylglucosides by Fischer,[14] a large number of investigators have found different values for the ratio k_α/k_β in the acid hydrolysis of these methylglucosides. Following the value of 3 given by Ekenstein,[1] a

[12] Moelwyn-Hughes, *Trans. Faraday Soc.*, 1929, **25**, 503.

[13] This is in contradiction to the results of Armstrong and Caldwell.[3]

[14] Fischer, *Berichte*, 1893, **22**, 1464.

value of 2.42 was found to hold at 70°C. by Freudenberg, Dürr and Hochstetter;[8] the ratio from the data of Armstrong and Caldwell[3] at 75°C. is 1.91. The general discrepancy between the various values which have been recorded for this ratio is seen from the above to be a consequence of the fact that the hydrolyses of the two isomeric methylglucosides have different critical increments.

A summary of the critical increments for the hydrolysis of some of the simpler carbohydrates by hydrochloric acid is given in the table. The data for lactose, sucrose, melezitose and raffinose are due to Stothart;[15] the value for maltose is that found by Kieran;[15] and the critical increments in the case of mandelonitrile glucoside and gentiobiose have been calculated from the results of Caldwell and Courtauld on the hydrolysis of amygdalin.[16] It is clear from the values of the critical increments (E) given in this table that the assumption made by Kuhn and Sobotka[6] in regarding the ratios $\frac{k_{\alpha\text{-methylglucoside}}}{k_{\text{maltose}}}$ and $\frac{k_{\beta\text{-methylglucoside}}}{k_{\text{salicin}}}$ as being independent of temperature is not permissible since E for α-methylglucoside is not equal to E for maltose, and the E values for β-methylglucoside and salicin are different. Both these ratios increase with rise in temperature. Armstrong and Glover,[4] on the other hand, are justified in assuming $\frac{k_{\text{sucrose}}}{k_{\text{raffinose}}}$ to be unaltered by temperature, for subsequent work has shown that the critical increments for the hydrolysis of these two sugars are the same. The same remark applies to the work of Josephson[7] on the hydrolysis of salicin and arbutin.

The considerations given above serve to emphasise the importance of obtaining critical increment data in the study of hydrolytic reactions, particularly when the object of the research is the comparison of acidic and enzymic hydrolyses, for these two must necessarily be investigated at different temperatures. Incidentally it may be remarked that, in the study of the hydrolysis of glucosides by acids, the critical increment has been found to be a much more significant quantity than velocity itself. It is not improbable that the same may

[15] Unpublished work carried out in this Laboratory, 1922–26.

[16] Caldwell and Courtauld, *Trans. Chem. Soc.*, 1907, **91**, 666.

be true of the hydrolysis of glucosides by enzymes, although there are obvious experimental difficulties in the way when the true critical increment for the enzymic hydrolysis of a given substrate is desired.

SUMMARY

Attention is drawn to the necessity of taking the values of the critical increments into account when the velocity constants for reactions of any pair of glucosides are compared. The ratio of the velocity coefficients for the hydrolysis of any two glucosides, determined at the same temperature, varies with temperature except in the special case when both hydrolyses have the same critical increment. Different values given by various investigators for the same ratio are shown to be due to the fact that comparative experiments have been carried out at different temperatures with two glucosides possessing different critical increments of hydrolysis. In the light of these considerations it becomes necessary to revise certain deductions which have been drawn from the comparison of rates of hydrolysis of glucosides by acids (at fairly high temperatures) with the rates of hydrolysis of glucosides by enzymes (at relatively low temperatures).

The writer is indebted to the Department of Scientific and Industrial Research of the British Government for a maintenance grant which has enabled him to carry out this work (as Research Assistant to Professor W. C. M. Lewis, F.R.S.), and to Imperial Chemical Industries Limited for a grant made to the Department of Physical Chemistry of this University.

THE KINETICS OF ENZYME REACTIONS: SCHÜTZ'S LAW

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Emil Schütz,[1] who followed polarimetrically the course of the peptic digestion of globulin-free albumin, discovered that the quantity x of albumin digested in a given time t was proportional to the square root of the amount e of pepsin employed:

$$x = \text{const.}\ \sqrt{e} \qquad (1)$$

Borissov[2] and Samojloff,[3] who independently arrived at the same result for tryptic and peptic digestion respectively, showed in addition that when the quantity of enzyme employed was maintained constant the extent (x) of digestion was proportional to the square root of the time:

$$x = \text{const.}\ \sqrt{t} \qquad (2)$$

These joint observations may be written in the form:

$$x = k_s \sqrt{e\,t}$$

or

$$k_s = \frac{x}{\sqrt{e\,t}} \qquad (3)$$

which is known as Schütz's Law. A repetition of the work of Emil Schütz by Julius Schütz,[4] who estimated the quantities of peptone produced by means of nitrogen determinations after precipitation of unchanged protein, substantially confirmed the conclusions arrived at by the earlier investigator, but indicated that the rule is approximate

[1] Euler, *Chemie der Enzyme*, Munich, 3rd edition, 1925, **2**, 511.
[2] Euler, General Chemistry of the Enzymes, New York, 1st edition, 1912, 175.
[3] Euler, General Chemistry of the Enzymes, New York, 1st edition, 1912, 175.
[4] Schütz, J., *Z. physiol. Chem.*, 1900, **30**, 1.

only, since with high concentrations of enzyme the quantity of protein digested is lower than that demanded by equation (3). Arrhenius,[5] analysing the electrical conductivity data of Sjöqvist[6] on the peptic digestion of egg albumin, concludes that Schütz's Law in this case holds for the first half of the reaction. Schütz's Law has been found to hold by Vernon[7] for the tryptic digestion of fibrin, by Engel[8] for the action of pancreatin on egg yolk emulsion, by Rubner[9] for the fermentation of 20 per cent cane sugar solutions, determined calorimetrically, and by Arrhenius[10] for the action of steapsin on fats. The data of Armstrong[11] on the hydrolysis of lactose by emulsin conform better with the Schütz equation (3) than with the ordinary unimolecular velocity constant equation, particularly when the concentration of emulsin is low. In more recent years W. van Dam[12] has found that equation (1) holds for the peptic hydrolysis of casein, while Northrop[13] finds that when the quantity of substrate is high and the amount of enzyme employed is low, Schütz's Law holds for the hydrolysis of peptone by pepsin. A similar behaviour is encountered in the case of the tryptic hydrolysis of casein.[14] Willstätter, Waldschmidt-Leitz, Duñaiturria and Künstner,[15] show that, when the quantity of enzyme is small in comparison with that of the substrate, Schütz's Law holds for the hydrolysis of casein by trypsin-kinase, provided the early stage of the reaction is neglected.

In general it may be concluded that Schütz's Law is an empirical relation which has been found to have a wide applicability to data on enzyme reactions, particularly when the concentration of enzyme is

[5] Arrhenius, Immunochemie, Leipsic, 1st edition, 1907, 53.

[6] Euler, *Chemie der Enzyme*, Munich, 3rd edition, 1925, **2**, 512.

[7] Vernon, *J. Physiol.*, 1901, **26**, 421.

[8] Euler, *Chemie der Enzyme*, Munich, 3rd edition, 1925, **2**, 21.

[9] Rubner, *Chem. Centralblatt.*, 1905, **76**, 39.

[10] Arrhenius, Quantitative Laws in Biological Chemistry, London, 1st edition, 1915, 46.

[11] Armstrong, *Proc. Roy. Soc. London, Series B*, 1904, **73**, 507.

[12] van Dam, W., *Z. physiol. Chem.*, 1912, **79**, 247.

[13] Northrop, *J. Gen. Physiol.*, 1920, **2**, 471.

[14] Northrop, *J. Gen. Physiol.*, 1923–24, **6**, 723.

[15] Willstätter, Waldschmidt-Leitz, Duñaiturria, and Künstner, *Z. physiol. Chem.*, 1926, **161**, 191.

relatively small compared with that of the substrate, and when the temperature of investigation is maintained low, so as to minimise any complications introduced by the spontaneous inactivation of the enzyme. Differentiating equation (3) with respect to t, it is found that the relation may be written:

$$\frac{dx}{dt} = \frac{k_3^2 \cdot \epsilon}{2x} \qquad (4)$$

i.e. Schütz's Law implies that the rate of reaction is directly proportional to the concentration ϵ of enzyme, inversely proportional to the concentration x of products, and independent of the concentration of substrate.

A Theoretical Deduction of Schütz's Law on the Basis of the Law of Mass Action

The deduction of Schütz's Law given here is due originally to Arrhenius;[16] it is quoted by Euler[17] and is restated by Northrop[14] as follows:

When the concentration of enzyme varies as a result of some cause other than the spontaneous inactivation of the enzyme we have:

$$\frac{dx}{dt} = k\,(A - x)\,Q \qquad (5)$$

where A is initial concentration of substrate, x is the quantity which has undergone hydrolysis in time t; Q the quantity of free (active) enzyme is a function of time. Considering the following equilibrium between enzyme and products of hydrolysis, ϵ being the *total* enzyme content:

$$\text{Enzyme} + \text{Inhibitor} \rightleftharpoons \text{Enzyme-Inhibitor (complex)}$$

$$[Q] \qquad [x - (\epsilon - Q)] \qquad\qquad [\epsilon - Q]$$

$$K = \frac{[Q]\,[x - (\epsilon - Q)]}{[\epsilon - Q]}$$

[16] Arrhenius, *Medd. Nobel. Inst.*, 1908, **1**, 1.

[17] Euler, *Chemie der Enzyme*, Munich, 3rd edition, 1925, **1**, 154.

Arrhenius[16] shows that when $x \gg \epsilon$, this equation can be written in the form:

$$K = \frac{Qx}{\epsilon} \quad \text{or} \quad Q = \frac{K\epsilon}{x} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (6)$$

substituting this value of Q in equation (5) we arrive at the relation:

$$\frac{dx}{dt} = k \; K\epsilon \; \frac{A - x}{x} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

on integration:

$$k K \epsilon = \frac{1}{t}\left\{A \ln \frac{A}{A - x} - x\right\} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (8)$$

From equation (7) it is seen that the rate is inversely proportional to the concentration of products; hence this equation cannot be expected to hold during initial stages of the reaction. Since the concentration of substrate is high, it can be assumed that $(A - x)$ is roughly constant over a certain range, hence equation (7) reduces to the following approximation:

$$\frac{dx}{dt} = k \,.\, K\epsilon \,.\, \frac{A}{x} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (9)$$

Integration of this expression shows that:

$$\sqrt{2k \,.\, KA} = \frac{x}{\sqrt{\epsilon t}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (10)$$

which is Schütz's Law. Arrhenius has thus shown that Schütz's Law is but a modified form of the unimolecular law and can be derived on the assumptions that (a) the concentration of substrate is much greater than the concentration of enzyme, (b) heat inactivation of the enzyme does not play an appreciable part in altering the active enzyme content, (c) products of hydrolysis form a complex with the enzyme, and this inhibits further hydrolysis, and (d) the concentration of substrate remains sensibly constant throughout the range of reaction considered.

It should, perhaps, be pointed out that since Schütz's Law (equation 10) is but an approximate form of the more general equation

(8), it must follow that any reaction which obeys Schütz's Law, must also obey the Arrhenius equation (8). This has been found to be true in certain cases where both equations (8) and (10) have been applied to the experimental data. Arrhenius found this to be so for the saponification of ethyl acetate by ammonia.[10] Bayliss[18] also found equation (8) to hold for the tryptic digestion of casein and gelatin. Northrop[14] compares the applicability of the Schütz Law and of the Arrhenius equation to data obtained by him for the hydrolysis of casein by trypsin.

A Theoretical Deduction of Schütz's Law on the Basis of the Adsorption Theory

The deduction of Schütz's Law on the basis of the theory of adsorption is due originally to Langmuir,[19] whose treatment is followed here with certain modifications and extensions to cover stages of enzymic hydrolysis other than those represented by Schütz's Law.

Enzymes are regarded as colloids on the surface of which chemical reaction takes place. Consider the catalytic change $A \rightarrow B$. Let Θ_A and Θ_B be the areas of the enzyme surface covered with molecules of A and B respectively. Both Θ_A and Θ_B are, in general, functions of time and depend on the extent of the reaction. As used by Langmuir the limiting value of each such term is unity. The rate of reaction will be, in general, proportional to Θ_A, hence

$$\frac{dx}{dt} = k_L \theta_A \qquad (11)$$

It is to be noted that k_L is a proportionality factor measuring the average "reactivity" per adsorbed molecule, and is not necessarily the same as Langmuir's term ν_A, which is the rate of desorption of molecules of A from the enzyme surface. Considering a special case in which even at low bulk concentration of A, its adsorbability is so great that it covers practically the whole of the enzyme surface, Θ_A would be nearly unity, and would remain constant over a wide range of change in bulk concentration, *i.e.* the observed rate, $\frac{dx}{dt}$, of reaction would remain constant through the range of bulk concentration

[18] Euler, *Chemie der Enzyme*, Munich, 3rd edition, 1925, 2, 477.
[19] Langmuir, *J. Am. Chem. Soc.*, 1916, 38, 2221.

considered, thus giving a zero order reaction. It has not been possible to find any data on enzyme action to substantiate this point, probably because the concentrations of substrate necessary for the conditions which would give a zero order reaction are too low to allow of accurate measurements. Waldschmidt-Leitz,[20] referring to the data of Michaelis and Davidsohn[21] on the hydrolysis of sucrose by saccharase, states that a "rule which might be expected in a purely catalytic reaction does not appear to hold here, namely, the non-dependence of reaction velocity upon the concentration of substrate."

Clearly, however, this condition is not that under which the Schütz behaviour is observed. Some disturbing circumstance is occurring in the latter case and this is taken by Langmuir to be due to a displacement of A molecules by B molecules. As a consequence of this, Θ_A, instead of remaining constant, diminishes during the reaction, but its variable area is still independent of the bulk concentration of A, because the variation in Θ_A is due to the operation of B molecules, an independent factor.

Obviously the displacement effect produced by the B molecules cannot sensibly operate right at the beginning; we have therefore to consider a suitable stage in the process. The assumption that B molecules can displace A molecules means that the adsorbability of B must be still greater than the adsorbability of A in spite of the latter being taken (necessarily) as highly adsorbable. At the end of the process the whole surface must be closely packed with B molecules.

When we are in the stage corresponding to the Schütz conditions we have the Langmuir equation holding, *i.e.*

$$\alpha_B \theta_A \mu_B = \nu_B \theta_B \qquad (12)$$

where μ_B is rate of adsorption of B molecules.

ν_B is rate of desorption of B molecules.

α_B is, in general, nearly unity.

This equilibrium must be attained very rapidly compared with the rate of chemical change $A \rightarrow B$. This is implied in the assumption

[20] Waldschmidt-Leitz, Enzyme Actions and Properties, London, 1st edition, 1929, 27.

[21] Michaelis and Davidsohn, *Biochem. Zeit.*, 1913, **49**, 333.

regarding the excessively high adsorbability of B molecules. The equilibrium represented by equation (12) is therefore constantly maintained although Θ_A is diminishing, and Θ_B is increasing with time. (This is possible with rapid adjustment since μ_B is increasing with time.) Combining equations (11) and (12) we get:

$$\text{chemical rate} = \frac{k_L \nu_B \theta_B}{\alpha_B \mu_B} = k_L \left(\frac{\nu_B}{\alpha_B}\right) \frac{\theta_B}{\mu_B} \quad \ldots\ldots\ldots\ldots\ldots\ldots (13)$$

i.e. the rate is directly proportional to Θ_B, inversely proportional to μ_B (*i.e.* concentration of B), and independent of concentration of A. If the chemical reaction has progressed sufficiently Θ_B will approach unity and when this is approximately the case Schütz's expression holds.

It is thus possible to deduce Schütz's Law theoretically by applying Langmuir's theory of heterogeneous catalysis to the conversion of A molecules to B molecules under the influence of an enzyme, provided (a) both A and B molecules are highly adsorbable, (b) the adsorbability of B molecules is greater than that of A molecules, and (c) the initial stages of the reaction are not considered.

The True Critical Increment for Enzymic Hydrolysis as Calculated from the Schütz Empirical Constant k_s

The following section is intended to direct attention to a possible error which may be introduced into the calculation of the critical increment, E, for enzymic reactions if the experimental values of the Schütz constant (k_s of equation (3)) are used in the well known equation

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}.$$

A comparison of equations (3) and (10) or of the differential equations (4) and (9) shows that:

$$k_s^3 = 2 k K A \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (14)$$

where k_s = the Schütz empirical constant, determined experimentally by means of equation (3),

k = the true (hypothetical) unimolecular velocity constant for the enzymic reaction,

K = equilibrium constant governing complex formation,
and A = initial concentration of substrate.

It follows that:

$$2\frac{d\ ln\ k_s}{dT} = \frac{d\ ln\ k}{dT} + \frac{d\ ln\ K}{dT}$$

since A is independent of temperature. If each of these terms is multiplied by RT^2 we have the critical increments and heat effect of the processes to which k_s, k and K refer, *i.e.*

$$2\ E_s = E_{uni} + Q_v$$

or

$$E_{uni} = 2\ E_s - Q_v \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (15)$$

i.e. the true critical increment for the hydrolytic process is equal to twice the critical increment value calculated from the Schütz constant k_s, minus the heat of decomposition of the enzyme-products complex. Generally we may assume that Q_v will be negligibly small compared with the two other terms involved, so that

$$E_{uni} = 2\ E_s \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (16)$$

It can readily be shown that the critical increment calculated from k_L (equation (11)) is a true value, identical with that calculated for the theoretical unimolecular constant k (equation (9)).

Application of the Foregoing Considerations to the Case of the Hydrolysis of Casein by Trypsin-Kinase

Experiments were carried out on the hydrolysis of casein by trypsin completely activated with enterokinase, under conditions for which Schütz's Law was found to be valid.

Preparations

(a). *Enzyme Extract.*—Pigs' pancreas, free from fat, was prepared by drying with acetone and ether according to the procedure of Willstätter and Waldschmidt-Leitz.[22] 5 gm. of the dried gland powder were added to 100 cc. of a glycerol-water mixture containing 4 volumes of glycerol (B.D.H., A.R.*) to 1 vol. of dis-

[22] Willstätter and Waldschmidt-Leitz, *Z. physiol. Chem.*, 1923, **125**, 132.

*B. D. H. stands for British Drug Houses, Limited; A. R. stands for analytical reagent.

tilled water. The powder was dispersed through the liquid by shaking and the dispersion kept for 3 hours at 30°C. The finely divided suspension was centrifuged for about 90 min., and the supernatant liquid finally filtered. The clear extract thus obtained remains for some months without deterioration if kept in an ice chest.

(b). *Kinase Solution.*—Pig's intestinal mucosa was treated with acetone, acetone-ether mixture and with ether successively.[23] 2 gm. of the dried mucosa were dispersed in 100 cc. of 0.05 N ammonia and kept at 30°C. for 2 hours. The suspension was filtered, and the clear filtate evaporated to half its volume by means of a rapid current of air, warmed to 30°—35°C. The resulting solution was kept in the ice chest.

(c). *Casein Solution.*—A solution of casein was prepared by addition of 100 cc. 0.025 N ammonia to 6 gm. casein (Kahlbaum-Hammarsten), stirring and keeping for 1 hour at 30°C. At the end of this time the slight amount of insoluble matter was filtered off. The clear filtrate is referred to as a 6 per cent casein solution.

(d). *Buffer Solution.*—A mixture of equal volumes of N ammonia and N ammonium chloride was used as buffer mixture. The pH of this buffer is stated by Willstätter, Waldschmidt-Leitz, Duñaiturria and Künstner[15] to be 8.6 at 20° C., 8.9 at 30° C. The pH was measured at 20°C. with the glass electrode and found to be 8.6.

Experimental Procedure

The course of the tryptic hydrolysis of casein was followed according to the method described by Willstätter, Waldschmidt-Leitz, Duñaiturria and Künstner.[15]

An equal volume, always less than 1 cc., of enzyme extract was measured into each of a number of 50 cc. Jena glass flasks. This volume of enzyme extract was completely activated in each case by addition of 0.3 cc. kinase solution and distilled water to make the total volume 3 cc., and by leaving the contents for 30 min. at 30°C. At the end of this period of activation 2 cc. buffer solution and 5 cc. of 6 per cent casein solution were added, making the total volume of reaction mixture in each flask equal to 10 cc. At intervals reckoned from the instant at which the casein was added, the increase in acidity of the solutions was estimated by the Willstätter method of stepwise-titration, in 50 per cent and 90 per cent alcohol, using 0.2 N NaOH (prepared in 90 per cent alcohol). In each case a blank experiment was performed with casein and buffer alone for the particular time-interval, the enzyme and kinase being added only at the end of this period, immediately before titration. In the results tabulated below, the alkali titres of the blank experiments have been subtracted from the total titres so that x cc. of 0.2 N NaOH) in the tables is a measure of the actual increase in acidity of the casein solution due to the tryptic action.

The enzyme quantity, ϵ, is expressed in terms of the trypsin unit defined by Willstätter, Waldschmidt-Leitz, Duñaiturria, and Künstner,[15] preliminary work

[23] Waldschmidt-Leitz, *Z. physiol. Chem.*, 1923–24, **132**, 204.

having led to a confirmation of the definition given by these authors. The data obtained for the tryptic hydrolysis of casein, using the method described above, are summarized in Tables 1 and 2.

TABLE 1

Temp. 30°C.

Total Volume of Reaction Mixture 10 Cc., Containing 2 Cc. of Buffer Having pH 8.9 at 30° C.

ϵ = 0.5 trypsin unit			ϵ = 0.75 trypsin unit		
t	x (cc. of 0.2 N NaOH in 90 per cent alcohol)	$k_2 = \frac{x}{\sqrt{et}}$		x (cc. of 0.2 N NaOH in 90 per cent alcohol)	$k_2 = \frac{x}{\sqrt{et}}$
min.			*min.*		
20	0.56	(0.177)	20	0.80	(0.201)
30	0.86	(0.221)	30	1.14	(0.240)
40	1.14	0.254	40	1.34	0.253
60	1.35	0.247	50	1.57	0.256
80	1.62	0.256	60	1.67	0.248
100	1.74	0.246	80	1.97	0.254
120	1.92	0.248	100	2.14	0.247

TABLE 2

Temp. 40°C.

*Total Volume of Reaction Mixture 10 Cc., Containing 2 Cc. Buffer Having pH 8.9 at 30°C.**

ϵ = 0.60 trypsin unit			ϵ = 1.20 trypsin units		
t	x (cc. of 0.2 N NaOH in 90 per cent alcohol)	$k_2 = \frac{x}{\sqrt{et}}$		x (cc. of 0.2 N NaOH in 90 per cent alcohol)	$k_2 = \frac{x}{\sqrt{et}}$
min.			*min.*		
30	1.61	0.379	20	1.79	0.365
40	1.78	0.363	30	2.23	0.372
50	2.03	0.371	40	2.60	0.375
60	2.15	0.359	50	2.87	0.370
80	2.58	0.372	60	3.03	0.358

* The change in pH of the system due to an increase in temperature of 10° will be about 0.2 pH. This magnitude of change—*at the optimum pH region for the tryptic action on casein*—may be neglected, since it may be seen from the curves obtained by Northrop[24] that a change of 0.2 pH, round the optimum pH region, will not introduce any appreciable error into the rate of hydrolysis.

[24] Northrop, *J. Gen. Physiol.*, 1922–23, **5**, 263.

From the figures in Table 1 the average of 10 values for k_s at 30°C. is equal to *0.251*. The values given in brackets are somewhat lower than this mean, and refer to stages of the reaction for which Schütz's Law does not hold.

From the figures in Table 2 the average of 10 values for k_s at 40°C. is equal to *0.368*. Substituting these values of k_s (0.251 at 30°C.; 0.368 at 40°C.) into the equation $\frac{d\ ln\ k_s}{dT} = \frac{E_s}{RT^2}$, it is found that the observed critical increment E_s is 7,200 calories. This, however, is an empirical value related to the true critical increment of hydrolysis according to equation (15). Employing the approximate relation of equation (16), it is found that E_{uni} becomes 14,400 calories.

It is to be noted that this value should be identical with that calculated from observed *unimolecular* constants for the tryptic hydrolysis of casein obtained at two temperatures. Unfortunately, however, values of unimolecular coefficients k_{uni} for this reaction have not been determined at two temperatures. Northrop[25] has succeeded in obtaining a unimolecular constant for the reaction at 0°C., but no temperature coefficient is recorded. The difficulties in the way of determining the value of E_{uni} have been (1) the necessity of accurate temperature control at low temperatures, and (2) the correct evaluation of A, *i.e.* the total extent of reaction. An attempt is being made at present to investigate this point more fully.

Waldschmidt-Leitz[26] has pointed out that the data available on the effect of temperature on enzymic reactions are largely taken from the older work where the significance of H^+ ion concentration, for example, was not realised, and consequently it is difficult to obtain data with which the results cited in this paper may with certainty be compared.

Vernon [26] in a study of the hydrolysis of Witte's peptone by trypsin, found that the time required to digest a given percentage of the peptone varied inversely as the quantity of enzyme employed, *i.e.*

$$et = \text{const. for a constant value of } x.$$

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[25] Northrop, *J. Gen. Physiol.*, 1923–24, **6**, 417.
[26] Vernon, *J. Physiol.*, 1904, **30**, 330.

Thus it is not clear whether the Schütz Law or the unimolecular relation is the significant expression for the reaction as carried out by Vernon.

From Vernon's data, however, it is possible to calculate a value of E_s which is 7,150 calories, a value which is in agreement with that obtained in the present work. The value calculated by Euler is 14,300 calories, on account of the fact that he substituted the values of t, at the two temperatures, in the equation for the critical increment, instead of the value of $\sqrt{t}$ at the two temperatures.

It is interesting to observe that Auld[27] finds a value for E_{uni} of 14,670 calories for the hydrolysis of salicin by emulsin.

SUMMARY

1. A review of the applicability of Schütz's Law to enzymic reactions is given.

2. The theoretical deductions of the Law, (a) on the basis of the law of mass action, (b) on the basis of the adsorption theory, are given and the significance of the assumptions made in these deductions pointed out.

3. It is shown that the true critical increment for an enzymic reaction is equal to twice the critical increment calculated from the Schütz constant k_s, if the heat of decomposition of the enzyme-products complex be neglected.

4. Experiments are described on the tryptic hydrolysis of casein at 30°C. and 40°C. The foregoing considerations are applied to the experimental results obtained.

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[27] Auld, *Trans. Chem. Soc.*, 1908, 93, 1275.

OSMOSIS OF LIQUIDS. III

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INTRODUCTION

In the preceding communications[1] we have discussed some of the phenomena presenting themselves during osmosis when the membrane either transmits a single substance or when it is permeable for all substances. Before discussing the results of other experimentally determined systems, the deduction of the direction of diffusion etc., we shall first consider from another point of view some of the phenomena, which may be met with during osmosis.

1. The Membrane is Permeable for a Single Substance Only

We take two liquids L and L' which both contain water and besides an arbitrary number of other substances, which may or may not be different in the two liquids. We now imagine in the osmotic system:

$$L \mid L' \qquad (1)$$

a membrane $M(W)$, *viz.* a membrane permeable for W(W = water). For this system we will then obtain, among other things, as we have seen in Paper I: (1) it depends upon the O.W.A. (Osmotic-Water-Attraction) of both the liquids whether water will diffuse or not; (2) when the two liquids have the same O.W.A., then no water diffuses through the membrane; (3) when the two liquids have a different O.W.A., then water will diffuse towards that side of the membrane, where the O.W.A. is greatest; (4) with this osmosis the water may go through the membrane positively or negatively and the concentrations of the substances may change normally or abnormally; with all these phenomena, as we have seen before, the nature of the membrane $M(W)$ is of importance only with respect to the velocity with which

[1] Schreinemakers, F. A. H., *J. Gen. Physiol.*, 1928, 11, 701; 1929, 12, 555.

the water diffuses, not, however, with respect to the direction of the diffusion; this namely is determined only by the O.W.A. (*viz.* the difference) of the two liquids.

We may also deduce this quite simply in the following way. For this purpose we imagine between the liquids L and L' an impervious wall in which are two openings; in one opening we bring a membrane A and in the other a membrane B; we imagine that both these membranes are permeable for water only. We then get a system, which we shall represent by (2).

$$L \left| \begin{matrix} A \\ \\ B \end{matrix} \right. L' \qquad (2)$$

Now we imagine these membranes so far apart from each other, that they can function independently of one another. If the water now did not diffuse through both membranes in the same direction, but through A in one and through B in the other direction, then a "circular current" of water would arise in (2). When the quantities of water diffusing through these membranes differ, then L and L' change their compositions, so that at last the system comes in a state of equilibrium; then the circular current will disappear. If, however, we regulate the surfaces of the membranes in such a way, that through the one as much water will diffuse towards the left as through the the other towards the right, then L and L' do not change their compositions and we get an eternal circular current. As we assume, however, that this is not possible, we may conclude that the water must diffuse through both membranes in the same direction.

If, therefore, we have a membrane permeable for water only, it will be only the O.W.A. of the two liquids which determines the diffusion-direction of the water; the nature of the membrane only influences the velocity of the diffusion.

Everything we have discussed above for the osmosis of water also obtains for any other substance S through a membrane $M(S)$, *viz.* through a membrane which is permeable only for the substance S. It then depends only upon the O.S.A. (Osmotic-S-Attraction) of the two liquids as to whether the substance S will or will not diffuse, not upon the nature of the membrane $M(S)$; this only determines the velocity of the diffusion; when both liquids have the same

O.S.A., then no S diffuses through the membrane; when the liquids have a different O.S.A., then the substance S diffuses towards that side of the membrane, where the O.S.A. is greatest; the substance S may go positively or negatively through the membrane and the concentrations of the substances may change normally or abnormally.

In Paper I we have discussed the phenomena which may occur with the osmosis through a membrane permeable for water only. All this obtains also, however, for the osmosis of the substance X through a membrane $M(X)$, *viz.* a membrane permeable for the substance X only. In these considerations the O.W.A. of the two liquids must then be substituted by their O. X. A. and the isotonic W-curves

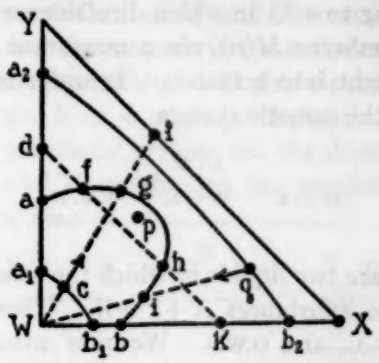


FIG. 1. This figure originally appeared as Fig. 2 in Paper I (*J. Gen. Physiol.*, 1928, **11**, 702).

by isotonic X-curves. An isotonic W-curve represents the liquids having the same O.W.A.; its two terminating points are situated on the sides WX and WY of the concentration-diagram (Fig. 1). An isotonic X-curve represents the liquids having the same O.X.A.; its two terminating points are situated, however, on the sides XW and XY of the diagram. Of course the same obtains also for the osmosis of the substance Y through a membrane $M(Y)$; then the O.W.A. of the two liquids must be substituted by their O.Y.A. and the isotonic W-curves by isotonic Y-curves, the terminating points of which are now situated on the sides YW and YX of the diagram.

2. The Membrane is Permeable for All Substances. Congruent and Incongruent Osmosis

When in a system we insert a membrane permeable for a single substance only, then the direction in which this substance will diffuse is independent of the nature of the membrane. If, however, we bring a membrane into that system, permeable for all substances, then, as we shall see further on, the nature of the membrane will indeed play a part; already in Paper II some examples have shown us that the form of the osmosis-path of a system is dependent upon the membrane; in following communications we shall discuss some more examples of it.

We are now going to ask: in which directions will the substances diffuse, when a membrane $M(n)$, *viz.* a membrane permeable for all substances, is brought into a system. In order to concentrate our thoughts, we take the osmotic system:

$$\begin{array}{ccc} & L \mid L' & \\ \text{O. X. A.} & \text{O. Y. A.} & \text{O. W. A.} \\ \longleftarrow & \longrightarrow & \longrightarrow \end{array} \qquad (3)$$

in which L and L' are two liquids of which the composition is known and containing the substances $X+Y+W$. These liquids have a definite O.X.A., O.Y.A., and O.W.A. We now assume that the left-side liquid has a greater O. X. A. than the right-side liquid; this has been indicated in (3) by the first arrow, as this points towards that side of the membrane where the O.X.A. is greatest. If we bring a membrane $M(X)$ in system (3), then the substance X will also diffuse in the direction of the first arrow, *viz.*, towards the left.

The second arrow indicates that we suppose that the O.Y.A. on the right side of the membrane is greater than on the left side; when we bring a membrane $M(Y)$ in the system, then the susbtance Y will diffuse also in the direction of the second arrow, *viz.*, towards the right.

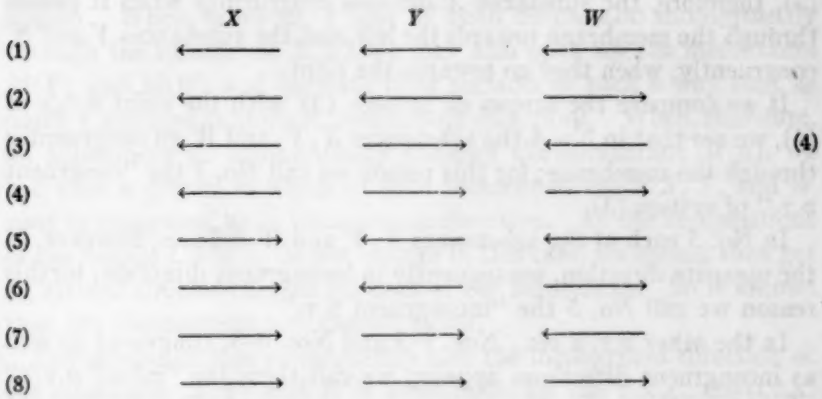
The third arrow indicates that we suppose that the O.W.A. on the right side is greater than on the left side; consequently, through a membrane $M(W)$ the water will also diffuse towards the right. Therefore, the arrows in (3) have two meanings, of which the one follows from the other, namely: they point towards that side of the

membrane, where the O.A. of a substance is greatest; they indicate the direction in which a substance diffuses through a membrane permeable for this substance only.

In system (3), therefore, the substance X will, according to our supposition, diffuse through a membrane $M(X)$ towards the left, the substance Y through a membrane $M(Y)$ and the water through a membrane $M(W)$ towards the right.

What will happen now when we bring a membrane $M(n)$ in system (3), so that all substances diffuse at the same time? Perhaps we might think that the substances must in this case also diffuse according to the arrows in (3), *viz.* from smaller towards greater O.A.; consequently X towards the left and Y and W towards the right; this is certainly possible, but not necessary; for in the case of this diffusion the nature of the membrane $M(n)$ will play a part.

If we omit the signs $>$ or $<$, and also the vertical arrows, and if we only consider the horizontal arrows, *viz.* the directions in which the substances X, Y, and W go through the membrane, then we may imagine eight cases, *viz.*:



We call each of these cases a D.T. (diffusion-type); consequently eight D.T.'s may be conceived. If, however, not only the diffusion-directions are considered, but also the velocities with which these substances diffuse, besides positive or negative and normal or abnormal osmosis, then each of the eight D.T.'s consists of an infinity of others. The relations existing between the velocities in these

D.T.'s will be left out of consideration here. In further discussions we are only going to consider the directions, so that we distinguish eight D.T.'s only.

In order to simplify a closer discussion of the subject, we shall first introduce another term; as it is we have already discussed positive and negative, normal and abnormal osmosis; now we shall distinguish in addition "congruent" and "incongruent" osmosis; that is, the substance X diffuses "congruently" through a membrane $M(n)$, when it passes through it in the same direction as through a membrane $M(X)$; consequently when X diffuses from smaller towards greater O.X.A. The substance X diffuses "incongruently" through a membrane $M(n)$, when it passes through it in the opposite direction than when going through a membrane $M(X)$; consequently when X diffuses from greater towards smaller O.X.A.

The same is also said for the substances Y and W and of course also for any other substance.

So the arrows in (3) have even a third meaning; they indicate also the congruent direction of each of the three substances. In system (3), therefore, the substance X diffuses congruently when it passes through the membrane towards the left, and the substances Y and W congruently, when they go towards the right.

If we compare the arrows of system (3) with the eight D.T.'s of (4), we see that in No. 4 the substances X, Y, and W go congruently through the membrane; for this reason we call No. 4 the "congruent D.T." of system (3).

In No. 5 each of the substances X, Y and W diffuses, however, in the opposite direction, consequently in incongruent direction; for this reason we call No. 5 the "incongruent D.T."

In the other D.T.'s *viz.*, Nos. 1–3 and Nos. 6–8, congruent as well as incongruent directions appear; we call them the "mixed D.T.'s" of system (3).

We are able to deduce thermodynamically: the incongruent D.T. is not possible; the seven other D.T.'s (*viz.* the congruent and the mixed ones) are possible, *i.e.* thermodynamically admissible; the nature of the membrane determines which of those seven admissible D.T.'s will occur.

We can prove in quite a simple manner that the incongruent D. T.

is not possible. For this reason we imagine an impermeable wall between the liquids L and L', in which there are four openings; into them we bring the membranes $M(n)$, $M(X)$, $M(Y)$ and $M(W)$. We then get a system which we shall represent by (5). We now imagine the three membranes $M(X)$, $M(Y)$, and $M(W)$ shut off at first, so that they cannot function. When the substance X diffuses incongruently through the membrane $M(n)$, *i.e.* in opposite direction as when diffusing through a membrane $M(X)$, we can open up the membrane $M(X)$ and regulate its surface in such a way that as much X runs through $M(X)$ in congruent direction as through $M(n)$ in incongruent

$$\begin{array}{l|l} & M(n) \\ L & \qquad L' \\ & M(X) \\ & M(Y) \\ & M(W) \end{array} \tag{5}$$

direction; then we get a circular current of the substance X. As, however, Y and W also run through the membrane $M(n)$, the liquids L and L' will change their compositions; as the system will at last reach a state of equilibrium, this circular current of X will also disappear. When, however, Y and W both diffuse also incongruently through the membrane $M(n)$, we may also open up the membranes $M(Y)$ and $M(W)$ and regulate their surfaces in such a way that as much Y and also as much W runs in both directions. When, therefore, all substances go incongruently through the membrane $M(n)$, we can form a system in which at each moment as much X, Y, and W runs in congruent as in incongruent direction. As the compositions of the liquids L and L' do not change in this case, we should then get an eternal circular current for each of the substances. So it follows that the incongruent D.T. cannot occur.

The congruent and consequently also the incongruent direction of the substances X, Y, and W is determined by the O.X.A., the O.Y.A. and the O.W.A. of the two liquids; these O.A.'s, however, depend upon the compositions of the liquids; if we change them, the O.A.'s will also change.

The direction of the arrows in (3) is consequently determined by the composition of the liquids L and L'; for a whole series of compositions the arrows in (3) will, therefore, keep the same direction; for other

compositions, however, one or more of these arrows will change their directions. If, *e.g.*, the O.Y.A. becomes greater on the left side of the membrane than on the right side, we have instead of (3):

$$\begin{matrix} \text{O. X. A.} & \text{O. Y. A.} & \text{O. W. A.} \\ \longleftarrow & \longleftarrow & \longrightarrow \end{matrix} \qquad (6)$$

It now appears from (4) that the D.T. No. 2 is congruent and No. 7 is incongruent. When after a further change in the composition of the liquids the O.X.A. becomes smaller on the left side than on the right side, then (6) passes into:

$$\begin{matrix} \text{O. X. A.} & \text{O. Y. A.} & \text{O. W. A.} \\ \longrightarrow & \longleftarrow & \longrightarrow \end{matrix} \qquad (7)$$

so that now D.T. No. 6 is congruent and No. 3 incongruent.

So it depends upon the composition of the liquids, which D.T. is incongruent and consequently not possible;[2] so we may say: the composition of the two liquids determines which of the eight D.T.'s is incongruent and, therefore, not possible; the nature of the membrane determines which of the seven other D.T.'s will occur.

Briefly we shall express this by saying: with the osmosis through a membrane $M(n)$ everything is possible, except all substances going incongruently through the membrane at the same time. Therefore, the nature of the membrane can have a great influence on the D.T. of a system; we shall briefly discuss some examples in which we have also been able to show this influence experimentally. We represent one of these results by:

	$X = NaCl$	$Y = Na_2CO_3$	W	
D. T. No. 3	$\longleftarrow$	$\longrightarrow$	$\longleftarrow$	(8)
D. T. No. 7	$\longrightarrow$	$\longrightarrow$	$\longleftarrow$	

This means the following. We took two liquids of definite compositions, containing the substances $X = NaCl$, $Y = Na_2CO_3$ and $W =$ water. We found that these substances diffused through some membranes, according to D.T. No. 3, through some other membranes,

[2] We may show that the D. T.'s No. 1 and No. 8 never can be incongruent.

however, according to the D.T. No. 7. So the diffusion-direction of the NaCl here changes with the nature of the membrane; one of the diffusion-directions of the NaCl must be congruent and the other incongruent. However, as long as we do not dispose of a membrane permeable for NaCl only, it is not possible to find out which direction is the congruent and which the incongruent.

In (8) only the horizontal arrows of the two D.T.'s have been indicated; with the aid of the complete D.T.'s we can indicate all changes, occurring at this moment of the osmosis. We found, *e.g.*, for one group of membranes:

$$\begin{array}{ccccc} & X & & Y & W \\ \bullet\downarrow < \uparrow\bullet & & \downarrow > \uparrow & & \uparrow < \downarrow \\ \longleftarrow & & \longrightarrow & & \longleftarrow \end{array} \qquad (9)$$

an abnormal-abnormal-positive osmosis of the NaCl. With the second group of membranes we found:

$$\begin{array}{ccccc} & X & & Y & W \\ \bullet\downarrow < \uparrow\bullet & & \downarrow > \uparrow & & \uparrow < \downarrow \\ \longrightarrow\bullet & & \longrightarrow & & \longleftarrow \end{array} \qquad (10)$$

an abnormal-abnormal-negative osmosis of the NaCl.

This shows once more what we stated previously that from the change in concentration of a substance we cannot deduce the direction in which this has diffused through the membrane. For in (9) and (10) the X-amount on the left side of the membrane becomes smaller and on the right side it becomes greater; yet the substance X has diffused in (9) towards the left and in (10) towards the right. In the system: $X = Na_2S_2O_6$, $Y = BaS_2O_6$ and $W =$ water, we found the D.T.'s:

$$\begin{array}{lccc} & X & Y & W \\ \text{D. T. No. 3} & \longleftarrow & \longrightarrow & \longleftarrow \\ \text{D. T. No. 4} & \longleftarrow & \longrightarrow & \longrightarrow \end{array} \qquad (11)$$

for two liquids of definite composition.

These substances, namely, diffused through a membrane of collodion according to the D.T. No. 3 and through a membrane of collodion in which was a deposit of $Cu_2Fe(CN)_6$ according to the D.T.

No. 4. So here it is the diffusion-direction of the water which changes with the nature of the membrane used; through one of these membranes, therefore, the water diffused congruently, through the other incongruently.

In the system: $X = NH_4Cl$, $Y =$ ammonium succinate and $W =$ water we also found for two liquids of definite composition the two D.T.'s which have been indicated in (11).

The D.T. No. 3 occurred when membranes (*a*) of collodion, (*b*) of cellophan and (*c*) of a pig's bladder were used.

The D.T. No. 4 occurred with a membrane (*d*) of collodion in which a deposit of $Cu_2Fe(CN)_6$; (*e*) of parchment and (*f*) of a pig's bladder. As is apparent from (*c*) and (*f*) the water in the system diffuses through one pig's bladder towards the left and through the other towards the right. This contrasting behaviour will have its reason in the difference in their antecedents before they were being used as membranes, such as degreasing, etc.

Now we take the osmotic system:

$$\text{water} \mid L\ (\text{water} + \text{tartaric acid}) \tag{12}$$

in which on the right side of the membrane is a solution of tartaric acid of definite concentration. If we bring a membrane $M(W)$ in here, then we have the osmotic system:

$$\begin{array}{c} \text{water} \mid M(W) \mid L \\ \longrightarrow W \end{array} \tag{13}$$

in which the water, as is indicated by the arrow, will diffuse towards the right, namely towards the solution; so in this system the congruent direction of the water is towards the right.

If, however, we bring a membrane $M(n)$ in this system, then it will depend upon the nature of this membrane whether the water will diffuse towards the right or towards the left; as there is no tartaric acid on the left side of the membrane, this will of course diffuse towards the left. So we can distinguish the two D.T.'s:

$$\begin{array}{c} \text{water} \mid M(n) \mid L \\ \longrightarrow W \qquad \longleftarrow Z \\ \longleftarrow 0\,W \qquad \longleftarrow Z \end{array} \tag{14}$$

in which the tartaric acid has been represented by Z. The sign 0 indicates that the water diffuses incongruently in the second D.T. With a membrane of cellophan we found the first D.T.; for the water diffused through this membrane towards the solution. With a membrane made of a pig's bladder we found the second D.T.; for the water diffused, just as the acid, through this membrane from the solution towards the water, consequently in incongruent direction.

It appears from the examples discussed above, that in a given system it depends upon the nature of the membrane $M(n)$ whether one or more (not all) substances will diffuse incongruently; we may also imagine membranes, through which a certain substance in a given system does not diffuse, although the membrane is permeable for this substance.

Let us take *e.g.* the system (11) in which the water diffuses through some membranes towards the left and through other membranes towards the right. As transition we may also imagine a membrane, through which no water diffuses, so that we have the transition—D.T.:

$$\longleftarrow X \qquad \longrightarrow Y \qquad - W \qquad (15)$$

Then we have a system, in which at that moment no water passes through the membrane, although the membrane is permeable for water.

A similar phenomenon often occurs during the osmosis. If we leave a system alone, so that it will run along its osmosis-path, then it often occurs that one of the substances will diffuse towards the left in one part of this path and towards the right in the other part. So during the osmosis there must be a moment, in which one D.T. passes into the other and this substance does not diffuse, of course.

These considerations may also be extended to the case that n substances pass through the membrane; then we find: (1) there are 2^n conceivable D.T.'s; (2) the composition of the two liquids determines which D.T. is incongruent and, therefore, not possible; (3) the nature of the membrane determines which of the 2^n-1 other D.T.'s will occur.

From this it appears that the composition of the two liquids and the O.A.'s of the n substances resulting from them now only play a small part; all these O.A.'s together namely determine one single D.T.

only, *viz.* the incongruent one, consequently the D.T. which is not possible. The membrane, which has the choice of the 2^n-1 remaining D.T.'s now plays the important part here. If, however, we put $n=1$, *viz.* a membrane permeable for one single substance only, then the situation becomes altogether different. Then there are only $2^n = 2$ D.T.'s conceivable; the composition of the liquids determines which D.T. will be incongruent and, therefore, not possible. So now there remains only one D. T., so that the membrane has no choice any more. In accordance with the preceding we therefore find here also: in a given system a substance S will always diffuse through a membrane $M(S)$ in the same direction, *viz.* congruently; the nature of the membrane has no influence on this.

Let us imagine the case that there are six diffusing substances; then there are $2^6=64$ D.T.'s. If we take two liquids of definite compositions then 63 D.T.'s must still remain; it now depends upon the membrane which of those 63 D.T.'s will occur. If only we could find the membranes adapted for this, we perhaps might see these six substances diffusing in 63 different ways, and in each of these D.T.'s alternatively positive or negative and normal or abnormal osmosis may occur besides.

We must also take into consideration that the membrane is a colloidal substance, so that it may change its nature and, therefore, also its D.T., under the influence of all sorts of factors, *e.g.* the influence of the diffusing substances, age, hysteresis, etc. So we need not be surprised, when Nature, with the osmosis in vegetable and animal tissues, sometimes shows us an abundance of phenomena and a change of D.T.'s under all sorts of internal and external influences.

SUMMARY

If only one substance S passes through a membrane, the nature of this membrane is not of importance with respect to the direction of the diffusion; this is namely determined only by the O.S.A. of the two liquids.

If, however, more substances pass through a membrane, the nature of this membrane is of great importance.

If n substances diffuse through a membrane, we can distinguish 2^n cases, when we take into consideration only the direction in which

each of these substances passes through the membrane; if we call each of these cases a D.T. (diffusion-type), 2^n D.T.'s may be conceived. Now we can deduce: one of these D.T.'s is not possible, the other 2^n-1 D.T.'s are thermodynamically admissible. The composition of the two liquids determines which of the D.T.'s is not possible; the nature of the membrane determines which of the 2^n-1 other D.T.'s will occur.

OXIDATION-REDUCTION POTENTIALS AND THE POSSIBLE RESPIRATORY SIGNIFICANCE OF THE PIGMENT OF THE NUDIBRANCH CHROMODORIS ZEBRA*

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INTRODUCTION

The blue-purple pigment occurring in the blood and tissues of the nudibranch *Chromodoris zebra* Heilprin has been extensively studied by Crozier (1) who reported, among its chemical properties, certain reactions with oxidizing and reducing agents which indicated its ability to react reversibly and suggested its possible significance as a respiratory pigment. To establish more definitely the nature of the oxidation and reduction reactions and the mode of oxygen transfer, the pigment has been further investigated by means of the electrometric methods customarily employed in studying reversible oxidation-reduction systems.

A typical reversible oxidation-reduction system, similar to the ferric-ferrous or the ferricyanide-ferrocyanide systems, is formed by the blue-purple pigment and its yellow reduction product and the potentials obtained experimentally agree with those calculated from the standard oxidation-reduction electrode equation.

At the hydrogen ion concentration of the blood of the organism, the oxidation-reduction potential of the pigment is such that its participation in the chemical reactions of respiration seems possible.

Physical and Chemical Properties of the Blue-Purple Pigment

A brief summary of the properties of the pigment as given by Crozier (2), and as found experimentally, will aid in arriving at conclusions

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regarding the chemical nature of the substance and assist in understanding the experimental procedure.

A reversible acid-base transformation of the oxidized form occurs with a pK, as measured colorimetrically, of 4.3. The color in acid solution is an orange-red having a maximum absorption in the blue part of the spectrum beginning at 520 mμ and continuing into the violet. The color in moderately alkaline buffer solutions is blue-purple having a relatively sharp maximum at 595 mμ in the absorption spectrum as determined with a Keuffel and Esser color analyzer.

The blue-purple solution is dichromatic showing more blue by reflected light and more red by transmitted light.

Reversible oxidation-reduction changes occur for both the red-orange acid form and the blue-purple form, each being reduced to a yellow reductant by such reducing agents as hydrogen sulfide, zinc, sodium hyposulfite $Na_2S_2O_4$ (sometimes called also hydrosulfite), and being reoxidized by air, ferricyanide, or iodine. Putrefactive processes reduce the blue-purple to the yellow form (colorless in dilute solutions), from which the blue-purple form can be regenerated by atmospheric oxygen.

The blue-purple form is apparently stable to prolonged heating in neutral or slightly acid aqueous or alcoholic solutions, but is destroyed by heating in alkaline buffers or to dryness at 100°C., particularly when certain salts are present.

Methyl and ethyl alcohols containing water and aqueous buffer solutions readily form solutions of the blue-purple pigment in the concentrations usually obtained (about 0.001 N) but the pigment is insoluble in absolute methyl, ethyl, or isopropyl alcohols, in ethyl ether, ethyl acetate, acetone, chloroform, carbon tetrachloride, carbon disulfide, benzol, toluol, or petroleum ether. The red-orange acid form is more soluble in the alcohols and in ethyl ether than is the blue-purple.

Reoxidation by air of the reduced form in alkaline, neutral, or acid buffers is not inhibited by hydrogen cyanide or hydrogen sulfide even after boiling in the presence of a large excess of the reagent. No significant alteration is detectable on addition of sodium nitrite.

The solid material has not been isolated in pure condition.

Procedure

Purification of Extracts.—The organisms were immersed in distilled water containing chloroform or toluol and allowed to remain several weeks. Water soluble organic material, proteins, inorganic salts, and the blue-purple pigment were extracted. One or two washings of the hashed material served to extract most of the remaining pigment. The water solution was evaporated down on a steam bath until about one-third of its volume remained and an equal volume of methyl alcohol was added. Proteins and inorganic salts precipitated, and, if the volume was not too small, little adsorption of the pigment by the precipitate took place. The filtered solution was subjected to similar treatment until a relatively high degree of concentration was attained, care being taken not to destroy or alter the material by overheating or by too close an approach to dryness. Ether extraction may be useful to remove certain organic substances. The inorganic salts remaining are not harmful to the pigment but their effect on the pH of buffer solutions must be taken into account and corrected for, as the calcium and magnesium salts remove phosphates as well as exerting the usual salt effects.

To establish that no alteration of the pigment occurred during the process of purification, the absorption spectra of the purified solutions were compared with those of untreated water extracts (as determined by Dr. R. Emerson with a Koenig-Martens spectrophotometer) and found to be identical. No significant difference in the absorption spectra could be detected in material prepared from fresh, autolyzed, or partly putrefied organisms.

Preparation of the Sample for Titration.—The purified solution was added to about five times its volume of tenth molar phosphate buffer solution. A precipitation of phosphates, presumably of calcium and magnesium, sometimes occurred. After filtration, disodium phosphate was added to compensate for the phosphate removed. The pH of the buffer of the titrating solution was adjusted, by adding hydrochloric acid or sodium hydroxide, until within at least 0.03 pH of that of the solution that was to be titrated.

Determination of the pH.—Because of the presence of the oxidant and of small quantities of impurities likely to affect the accuracy of a hydrogen electrode, the pH was measured by the quinhydrone electrode. The oxidation-reduction potential zone of the pigment is of such order that quinhydrone is not appreciably affected by the oxidized form. Reproducible potentials were obtained which were constant for several minutes. The constant value was accepted as representing the pH of the solution, no correction being made for salt errors or liquid junction potentials, the latter being considered eliminated by the use of the saturated potassium chloride bridge material.

Apparatus and Method.—The potentials were measured by the usual potentiometric methods, employing a saturated calomel electrode as reference electrode. Calibration of the calomel cell was obtained by reference to the quinhydrone electrode in 0.05 M potassium acid phthalate. The value for this quinhydrone electrode was assumed to be $E_h = +0.456$ at 30°C.

Platinum electrodes were used for recording the oxidation-reduction potentials. The nitrogen used for deoxygenating the solutions and excluding oxygen from the burette was purified by passing through a heated tube containing copper.

TABLE I

Solution of Pigment Prepared from Partially Autolyzed Material, in Phosphate Buffer of pH 6.20. Titrated with Approximately 0.0006 N $Na_2S_2O_4$. *Final Concentration of Pigment Approximately 0.0003* N. *Temperature 30°C. ± 0.2°C.*

	E_h	E_h		E_h	E_h
cc.			*cc.*		
0.00	+0.2460	+0.2450	14.00	−0.0196	−0.0195
0.50	.2139	.2139	15.00	.0246	.0246
1.02	.1965	.1970	17.00	.0310	.0310
1.50	.1840	.1833	19.00	.0362	.0360
2.03	.1654	.1665	20.00	.0386	.0384
2.53	.1564	.1535	22.00	.0443	.0443
3.03	.1297	.1304	24.00	.0496	.0496
3.53	.1167	.1167	26.00	.0533	.0534
4.00	.1002	.1012	28.00	.0598	.0598
4.50	.0864	.0854	30.00	.0648	.0648
5.00	.0552	.0532	32.02	.0712	.0715
5.50	.0613	.0613	34.00	.0781	.0783
6.01	.0497	.0504	36.00	.0843	.0847
6.50	.0378	.0382	38.00	.0963	.0963
8.00	.0150	.0150	40.00	.1074	.1074
9.00	.0060	.0052	42.00	.1130	.1132
10.00	−0.0018	−0.0013	44.00	.1313	.1313
11.00	.0077	.0078	46.00	.1450	.1450
12.00	.0136	.0136	48.00	.160	.160
13.00	.0177	.0177			

The usual methods for determining the characteristic constant E_o',

$$\left(E_h = E_o' - \frac{RT}{nF} \log_e \frac{(\text{Red})}{(\text{Ox})}\right),$$

of a particular reversible oxidation-reduction system at constant pH, consist of measuring E_h while varying the concentration ratio of the reductant to oxidant by titrating the reductant with an oxidizing agent, by titrating the oxidant with a reducing agent, or by making up mixtures of the oxidant and reductant.

Because of a scarcity of the organisms and the small quantity of material available, the method most suited to this particular case, namely, the titration of the oxidant with the reducing substance, $Na_2S_2O_4$, was generally used. Titra-

tions of the reductant with potassium ferricyanide gave results similar to the titrations of the oxidant with $Na_2S_2O_4$, but because of lack of agreement of the electrodes in these solutions the results were excluded.

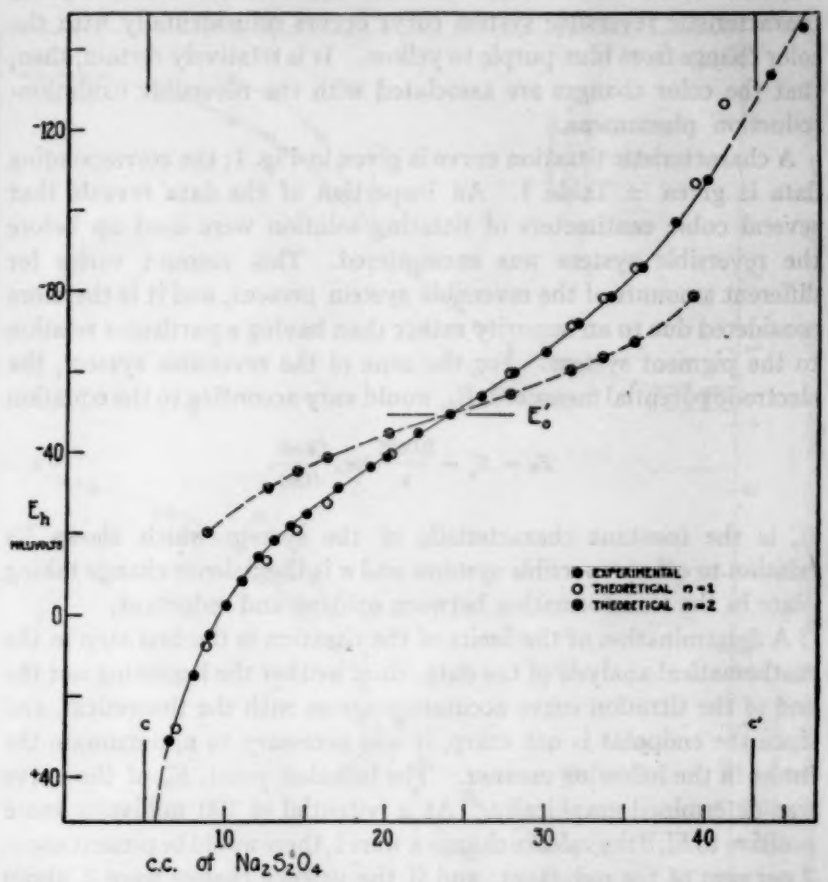

FIG. 1. Titration of pigment of *Chromodoris zebra* with $Na_2S_2O_4$. Phosphate buffer, pH 6.20; final concentration of pigment about 0.0003 N; temperature 30°C.

EXPERIMENTAL RESULTS

For the characterization of an oxidation-reduction system it is desirable to obtain information regarding the reversibility of the system, and, if the system be reversible, to evaluate the constants of the standard electrode equation. From the general shape of the

titration curves obtained, it was obvious that a reversible system is present in the solutions of the *Chromodoris zebra* pigment.

In all of the titrations made under the various conditions, the characteristic reversible system curve occurs coincidentally with the color change from blue-purple to yellow. It is relatively certain, then, that the color changes are associated with the reversible oxidation-reduction phenomena.

A characteristic titration curve is given in Fig. 1; the corresponding data is given in Table I. An inspection of the data reveals that several cubic centimeters of titrating solution were used up before the reversible system was encountered. This amount varies for different amounts of the reversible system present, and it is therefore considered due to an impurity rather than having a particular relation to the pigment system. For the zone of the reversible system, the electrode potential measured, E_h, would vary according to the equation

$$E_h = E'_o - \frac{0.0601}{n} \log_e \frac{(\text{Red})}{(\text{Ox})}.$$

E'_o is the constant characteristic of the system which shows its relation to other reversible systems and n is the valence change taking place in the transformation between oxidant and reductant.

A determination of the limits of the titration is the first step in the mathematical analysis of the data, since neither the beginning nor the end of the titration curve accurately agrees with the theoretical; and since the endpoint is not sharp, it was necessary to approximate the limits in the following manner. The inflexion point, E'_o, of the curve was determined graphically. At a potential of 100 millivolts more positive to E'_o, if the valence change n were 1, there would be present about 2 per cent of the reductant; and if the valence change were 2, about 1 per cent of reductant. The selection of either of these values as the per cent of conversion into the reductant at that potential would lead to no serious error. Having placed the asymptote C by this method, the position of the endpoint asymptote C' was fixed at the same distance from the inflexion point, E'_o, as C. A slight shift in the position of the asymptotes was made in some cases in order to obtain better fittings of the curve. With the inflexion point E'_o and the limits of the titration fixed, [(Red)/(Ox)] can be calculated from the titration

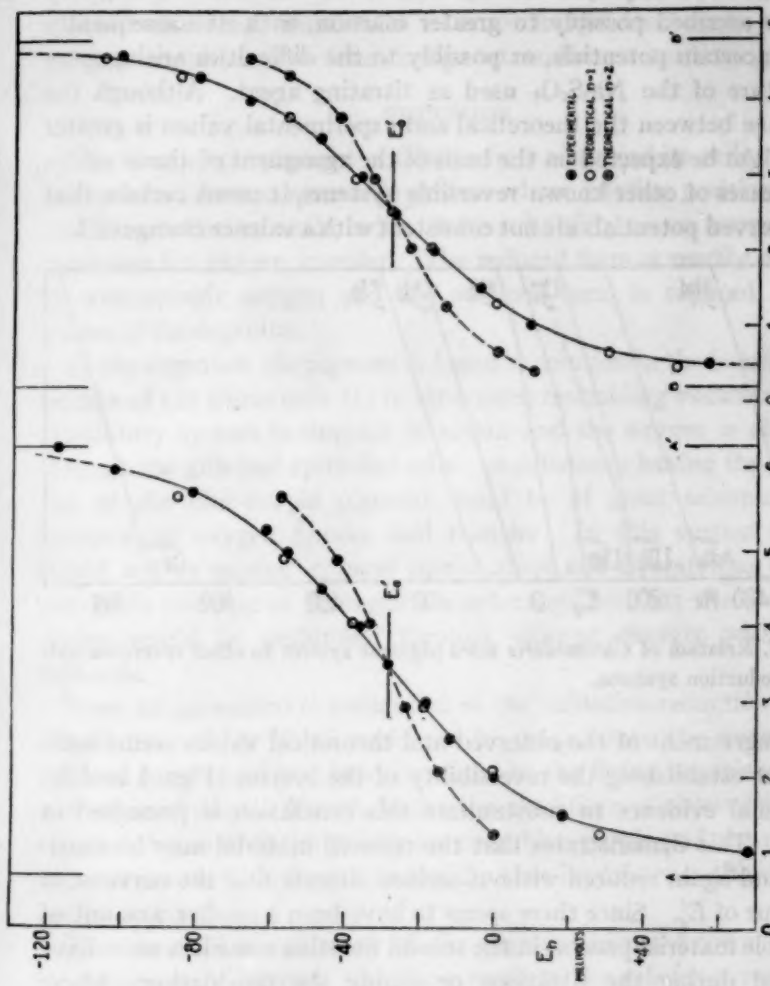

FIG. 2. Successive titrations of *Chromodoris zebra* pigment with $Na_2S_2O_4$ after reoxidation of the pigment by air. Phosphate buffer, pH 6.02; temperature 30°C.; final concentration about 0.0006 N.

volumes and the theoretical values of E_A for different values of n can be computed.

The theoretical values for $n = 1$ are in fair agreement with the experimental, except at the end of the titration. The discrepancy may be ascribed possibly to greater dilution, with its consequently more uncertain potentials, or possibly to the difficulties arising from the nature of the $Na_2S_2O_4$ used as titrating agent. Although the difference between the theoretical and experimental values is greater than might be expected on the basis of the agreement of these values in the cases of other known reversible systems, it seems certain that the observed potentials are not consistent with a valence change of 2.

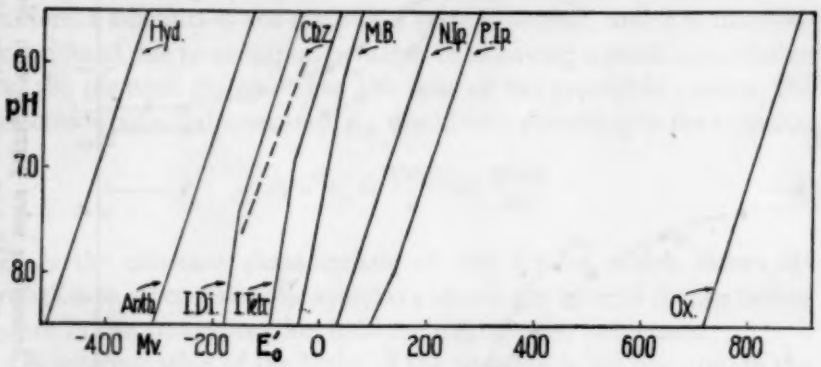

FIG. 3. Relation of *Chromodoris zebra* pigment system to other reversible oxidation-reduction systems.

The agreement of the observed and theoretical values seems sufficient for establishing the reversibility of the system (Figs. 1 and 2). Additional evidence to substantiate this conclusion is presented in Fig. 2. This demonstrates that the reduced material may be reoxidized and again reduced without serious alteration of the curve or of the value of E'_o. Since there seems to have been a smaller amount of titratable material present in the second titration some loss must have occurred during the titrations or during the reoxidation. Many reversible system reductants on oxidation by oxygen cause peroxidations which irreversibly oxidize some of the material to products other than the oxidant; similar processes may be operating in this case.

E'_o varies as the pH but not at one of the standard rates; however,

since such a short pH range was studied and nothing is known regarding the ionizations of the substances in this region, the exact relation of E_o' to pH must remain an open question. The results show definitely (Fig. 3) that in the vicinity of pH 7 the system lies in a position between the indigo disulfonate — leuco indigo disulfonate and the indigo tetrasulfonate — leuco indigo tetrasulfonate systems.

DISCUSSION

The blue-purple pigment of *Chromodoris zebra* and its yellow reduction product form a reversible system whose relative position in the oxidation-reduction scale indicates its suitability as an intermediary substance for oxygen transfer. The reduced form is readily oxidized by atmospheric oxygen and the oxidized form is reduced by the tissues of the organism.

In the organism the pigment is found in solution in the blood and in certain of the tissue cells (1) in structures resembling vacuoles. The circulatory system is sluggish in action and the oxygen is absorbed through the gills and epithelial cells. A substance having the properties of the blue-purple pigment could be of great advantage for accelerating oxygen uptake and transfer. In this system oxygen would not be carried in loose combination and liberated as oxygen gas, as in the case of hemoglobin-oxyhemoglobin, but the oxidizing action would be performed through valence changes within the molecule.

Even an approximate estimation of the oxidation-reduction potential of the cells or blood could not be made, since the quantity of reduced form could not be determined in the living organism under normal conditions. Always, under the conditions of observation, the exposed tissues of the organism are deep blue-purple and the reduced pigment reacts with oxygen so rapidly that its existence there would not be detected.

The chemical constitution of the pigment is not known but its properties are of interest. In many respects, the substance resembles an organic dye or coloring matter; in its color changes with pH and oxidation and reduction changes, in its absorption spectra, and in its non-reactivity towards cyanide and nitrite. It is unusual, however, to find an organic system in which $n = 1$; with the exception of

systems containing free radicals or complicated by the production of complexes of the quinhydrone (3) or meriquinoid types, n is always 2. Because of the small quantity of pigment available, the low concentration of the solutions used in the experiments, and the possible existence of traces of metals as impurities, qualitative tests for metals seemed without value and no attempt was made to test for the common metals having the proper valence change of 1.

A scarcity of material has necessitated the postponement of work on many of the interesting phases of this problem. As Crozier (1) has pointed out in his acid penetration studies with *Chromodoris zebra* tissues, the organism carries its own internal pH indicator, and, as the electrode potential studies have indicated, its internal oxidation-reduction indicator at the same time. The organism seems, therefore, particularly well adapted for use in the study of oxidation-reduction phenomena in the living organism without introducing foreign substances of possible toxicity. The isolation of the pigment and its chemical constitution will be studied, and the possible use of the substance or its related compounds as oxidation-reduction indicators awaits development.

The author wishes to thank Professor W. J. Crozier for the suggestion of the problem and his assistance in securing the biological material and wishes to express appreciation for his constant interest in the work. The author is also indebted to Professor J. B. Conant for his valuable advice and criticism.

SUMMARY

1. Oxidation-reduction potential methods have been applied to a study of the blue-purple pigment present in solution in the blood and in the tissue cells of the nudibranch *Chromodoris zebra*.

2. The blue-purple pigment and its yellow reduction product form a reversible system whose $E'_0 = -0.102$ volts at pH 7.0 and whose valence change from oxidant to reductant appears to be *one*.

3. The system is unlike oxyhemoglobin-hemoglobin in the mode of oxygen transfer. Its rôle as a possible respiratory material is discussed.

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THE DIRECT CURRENT RESISTANCE OF VALONIA

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I

The present paper, dealing with the direct current resistance of *Valonia*, is introductory to a series describing the use of this and other coenocytic algae (*Halicystis, Nitella, Chara*) in experiments involving the flow of electric current.[1] Such cells are as obviously adapted to these studies as they are to chemical and electrostatic measurements. Their advantages lie in the large and measurable surfaces, cell walls, and vacuoles, the effects of which may be better isolated than in tissues; and in the possibility of dealing with processes occurring across a single layer of protoplasm.

Besides resistance, there are other phenomena depending on the flow of current such as reactance and stimulation, which will be dealt with in other papers. Several years' work with these cells has corroborated the high importance of reactance in all these measurements. Changes of impedance in alternating current are strikingly great and occur at very low frequencies owing to the high capacitance of the protoplasm. The nature of this capacitance is more readily investigated in these cells where the capacitance is large and will be dealt with at length at a later time. The analogous direct current phenomena, the "transient" effects occurring at the opening and closing of the circuit, are large enough in magnitude and in duration to be recorded photographically. Finally, the phenomena of stimulation, and of injury and recovery, may be studied with definite control of current and potential difference across the surface in question.

[1] These might be grouped as "electrodynamic" or "electrokinetic" studies, but there are possible ambiguities in the use of either term. Likewise the phrase "secondary effects" (by analogy with secondary cells) has another connotation; see Rosenberg, H., *Handb. norm. u. path. Physiol.*, 1928, **8**, pt. 2, 926.

Fundamental to all these, however, both from the point of view of theory and of method, are the measurements of resistance to direct current in the steady state. All bioelectric measurements involve resistances inasmuch as the living system includes short circuits; and few experiments can be made under truly static conditions. This was shown in several cases by Osterhout and Harris[2] and by Damon.[3] Resistances must therefore be considered in any general bioelectrical theory, and in addition they give information *per se* as to the effective permeability of protoplasm to ions.

The methods described are used (with some adaptations of technical detail) in all the subsequent studies, and must frequently be employed with each cell to determine the value of leakage around the wall. The measurements are also of value in establishing the normal condition of the organism.

The experiments described in this paper are those performed with intact cells of *Valonia* in as nearly normal condition as possible, and with the application of direct potentials less than the minimal breakdown value.

II

The species used has been chiefly *Valonia ventricosa* J. Ag., of Florida. These cells reach volumes up to 30 cc., but are most useful in sizes about 2 cm. in diameter. They are not branched and are frequently nearly spherical or cylindroid, and very symmetrical. The wall is tough and only slightly extensible by ordinary pressures. The vacuolar sap is about 0.6 M with respect to chloride, of which nearly 95 per cent is KCl, the remainder being NaCl.[4] The cells live very well in the laboratory.

V. macrophysa, Kütz, is in general less useful for these studies on account of its branching and irregular shape. Only the smaller cells approach symmetry. The Bermuda organism is very hardy (except occasionally in the summer), but the *V. macrophysa* of Fort Jefferson, Dry Tortugas, Florida, while abundant and very large, does not live at all well in the laboratory, rarely surviving 2 weeks.

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[2] Osterhout, W. J. V. and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 679; *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 838.

[3] Damon, E. B., *J. Gen. Physiol.*, 1929–30, **13**, 207.

[4] Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, **68**, 164.

When zoospores are formed, the protoplasm is broken up and the wall is softened and punctured; while this can be delayed by addition of KCl to the sea water, it eventually claims most of the cells. I am inclined to question all results based on the use of this organism from Fort Jefferson. (See[4] for the presence of sulfate, calcium, and magnesium in the sap.)

There is considerable variation in the behavior of *V. ventricosa* when freshly collected, so that we prefer to reach a more uniform population by maintaining the cells under favorable conditions (diffuse light, and freedom from mechanical disturbance, in sea water). This may take surprisingly long—up to 2 or 3 months for large cells. During this time the cells are apparently healthy but display a low and variable resistance, which depends on the voltage applied.[5] As the cells remain in sea water, however, they slowly reach a uniformly high resistance over the range from 10 to 100 millivolts, and these are now considered to be "normal."

The variability is of intrinsic interest, but in view of the difficulties of establishing criteria of normality we were for a long time unable to assign this stage to its proper significance in the life of the organism. Recent experiments, however, with cells impaled on capillaries,[6] persuade us that it is in reality an effect of temporary injury, as previously surmised.

The source of this injury is to be sought in mechanical shock, to which *V. ventricosa* is extremely sensitive. The protoplasm evidently constitutes an unstable film which a slight prick frequently disrupts, breaking it into hundreds of tiny spheres—new cells which live and grow inside the old wall. The disturbances incident to collecting are often severe enough to produce this effect. The large cells grow among corals and sponges from which they must be separated and cleaned, at the risk of scratch and puncture by spicules, etc. Frequently a quarter to a third of the cells die within an hour after collection. The remainder are for some time a distinctly varied lot, rather useless for comparative purposes. Some have low, some high resistance; and they may even vary during the course of the experiment. Their variable permeability to ions in this state possibly accounts for the conflicting statements which have been made regarding the penetration of ionized substances into their vacuoles. Three facts suggest that this low and variable resistance is abnormal and due to injury: (1) there are cells found in nature the resistance of which is high and does not vary with potential. These are usually small cells, the least injured by mechanical shock. (2)

[5] Blinks, L. R., *Am. J. Physiol.*, 1928, **85**, 351.

[6] Blinks, L. R., *Carnegie Inst. Washington, Yearbook No. 28*, 1928–29, 277.

Only those cells which finally reach a high and steady value eventually survive. In general the larger a cell the longer it remains variable and the less are its chances of survival. (3) Cells which have reached a steady value may be made variable again by impalement on a capillary. They then tend to regain "normal" behavior while impaled but this is a slow process.

It is evident that length of life *after* the experiment is not a good criterion of condition *during* observation, since injury may be present, followed by recovery. The previous treatment is more significant than the subsequent history. For these reasons the present report deals with *V. ventricosa* kept long enough in the laboratory to reach a constant high resistance at low applied potentials.

III

The electrical circuit is a modified Wheatstone bridge using non-polarizable electrodes and a short period detector. This is indicated schematically in Fig. 1.

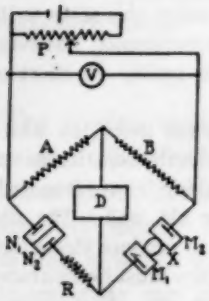

FIG. 1. Scheme of direct current bridge. P, potentiometer; V, voltmeter; A, B, equal ratio arms; D, detector; M_1M_2, N_1N_2, non-polarizable electrodes; R, variable resistance; X, cell.

In detail (Fig. 2) the bridge may be regarded as two potentiometers connected at or near equipotential points, the one, A, B, composed of equal ratio arms (pairs of 1000, or of 1,000,000 ohms); the other of the two parts: R (a variable decade resistance 0 to 1,000,000 ohms in 1 ohm steps); and X the object to be measured. From Y to Y' is connected the detector, actuated by the differences of potential existing when the bridge is not balanced.

The detector D is essentially another bridge in which two arms are the plate batteries B_1 and B_2 of equal potential drop (45 volts); and two others: the fixed resistance F (50,000 ohms); and the 201-A tube of nearly equivalent resistance under these conditions. The potentiometers q_1 (10,000 ohms) and q_2 (400 ohms) constitute slide wires for coarse and fine adjustment to balance the circuit so that no current flows in the string galvanometer G. This is done first with S_4 open leaving the grid free, then S_4 is thrown to the left and the grid bias adjusted at

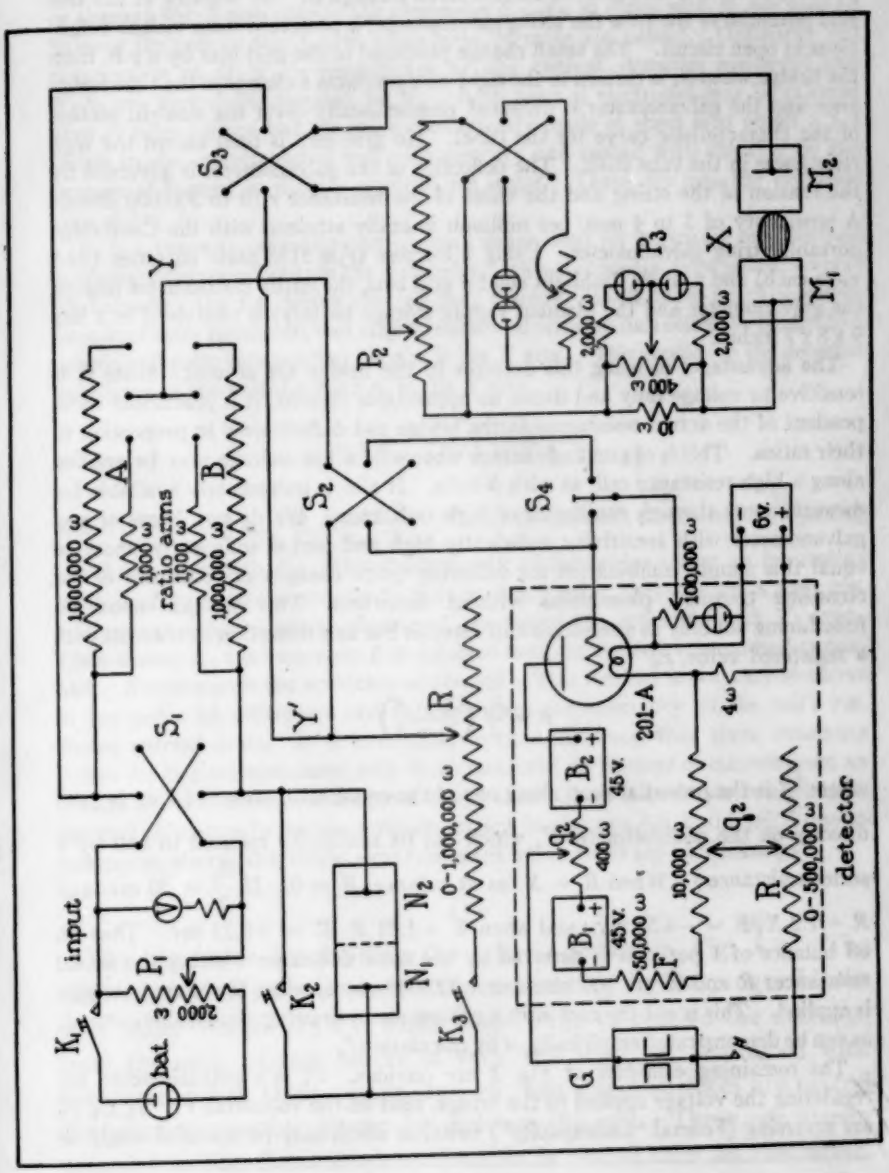

FIG. 2. Wiring diagram of bridge. Description in text.

potentiometer C until again no current flows through G. By working at the free grid potential of the tube the string galvanometer is protected from violent deflections in open circuit. The small change produced in the grid bias by a P.D. from the bridge when S_4 is thrown to the right now produces a change in the tube resistance and the galvanometer is deflected proportionally (over the straight section of the characteristic curve for the tube). No grid leak is used except the high resistances in the tube itself. The deflection of the galvanometer is governed by the tension of the string and the value of the resistance r (0 to 500,000 ohms). A sensitivity of 3 to 4 mm. per millivolt is easily attained with the Cambridge portable string galvanometer. Using 4 Burgess type 5158 plate batteries (22.5 volts each) and a single flashlight cell for grid bias, the entire electrometer (except the galvanometer and the filament supply storage battery) is contained in a box 9 x 8 x 7 inches.

The advantages of using this detector in the bridge are several. Since it is sensitive to voltage only and draws no appreciable current, it is practically independent of the actual resistances in the bridge and deflects only in proportion to their ratios. This is of great advantage when only a low voltage may be applied along a high resistance cell, as with *Nitella*. It also is immediately available for measurements through capillaries of high resistance. We do not know of any galvanometer with sensitivity sufficiently high and period sufficiently short to equal this simple combination for following quick changes of resistance or for recording transient phenomena without distortion. This depends upon its functioning not only as a sensitive null detector but as a deflection instrument with a registered value, E,

$$E = \frac{E_0}{2}\left(\frac{X - R}{X + R}\right)$$

where $\frac{E_0}{2}$ is the potential drop along one of the equal ratio arms. (This is read directly on the millivoltmeter V, which has its sensitivity reduced to half by a series resistance.) When $R = X$, as at balance, $E = 0$. If $\frac{E_0}{2} = 50$ mv. and $R = 1.2\,X$, $E = -4.55$ mv.; and when $X = 1.01\,R$, $E = +0.25$ mv. Thus an off balance of 1 per cent is detected by the same deflection whether the actual resistances R and X are 100 ohms or 1,000,000 ohms, when the same potential is applied. This is not the case with a galvanometer drawing appreciable current, as can be demonstrated empirically or by calculation.[7]

The remaining elements of Fig. 2 are obvious. P_1 is a potentiometer for regulating the voltage applied to the bridge, read on the voltmeter V. S_1, S_2, S_3 are reversing (Federal "anticapacity") switches which may be operated singly or

[7] Johnson, K. S., Transmission circuits for telephonic communication, New York, 1927, pp. 284–5.

together for reversing potential differences through the bridge and electrometer, or through the X arm only. The electrodes N_1, N_2, in series with R, are used to balance the pair in the X arm when large currents are passed. Ordinarily the polarization of M_1M_2 is insignificant at the current densities employed and N_1N_2 are kept short-circuited by K_2. Both pairs of electrodes may be calomel ones of large surface but are more conveniently made of sheet lead about 2 x 4 inches in size, amalgamated well with mercury and coated with chloride by the passage of current in slightly acidified sea water. These dip into the electrode cups of Fig. 3.

P_3 is a two-way potentiometer of small range used for balancing out residual potential differences between the electrodes. P_2 is a students' type potentiometer for balancing the P.D. which the cell may display. Both these potentiometers introduce extra resistance, and may be dispensed with by balancing the bridge to a pseudo null point representing the P.D. in the X arm. This records in the detector at balance as one-half the real P.D. in the X arm;

$$E = \text{P.D.} \left(\frac{A + R}{A + B + X + R}\right)$$

When $A = B$ and $X = R$, $E = \frac{\text{P.D.}}{2}$. It may be recorded in entirety by opening the R arm by switch K_3. This characteristic of the bridge allows an interesting method of balance comparable to that of Sen,[8] using only the cell's own P.D. Without an external source of current, we record the deflection with K_3 open. Then closing K_3 the resistance R is adjusted until the deflection is reduced to one-half. R now equals the resistance of the cell. This method is not very sensitive at low potential differences and depends upon the constancy of the cell's P.D. during current drain. It is interesting to note, however, that these resistance values are in good agreement with those measured by passage of current from an outside source of voltage of the same magnitude. There is no reason to anticipate any real differences in the two methods, except insofar as a P.D. in the cell is due to a chemical, electrical or mechanical treatment which alters the conductance.

IV

The apparatus for holding the cells is shown in Fig. 3 *a*. The electrodes M_1M_2 are in vessels communicating by large agar blocks with other vessels W_1W_2, which have wells to receive the drainage from the cells. Large blocks of very stiff agar gel, saturated with sea water, are in these vessels, and on their upper surfaces are placed the smaller movable blocks for holding the cell. These are shown enlarged in Fig. 3 *b*. A glass chamber is placed over all the latter,

[8] Sen, B., *Proc. Roy. Soc., London, Series B*, 1923, **94**, 216.

and the lower aperture *C* may be stuffed with non-absorbent cotton to keep out air currents.

Other methods of holding the cell were tried and abandoned. A cell wedged as closely as possible into a tube either is so loose as to allow the circulation of water past it or else the pressure on part of the surface is injurious. The latter is also the case when the cell is held in a hole punched in a thin sheet of rubber. The chloroplasts move away, and the cell becomes soft, changing its cross section, and

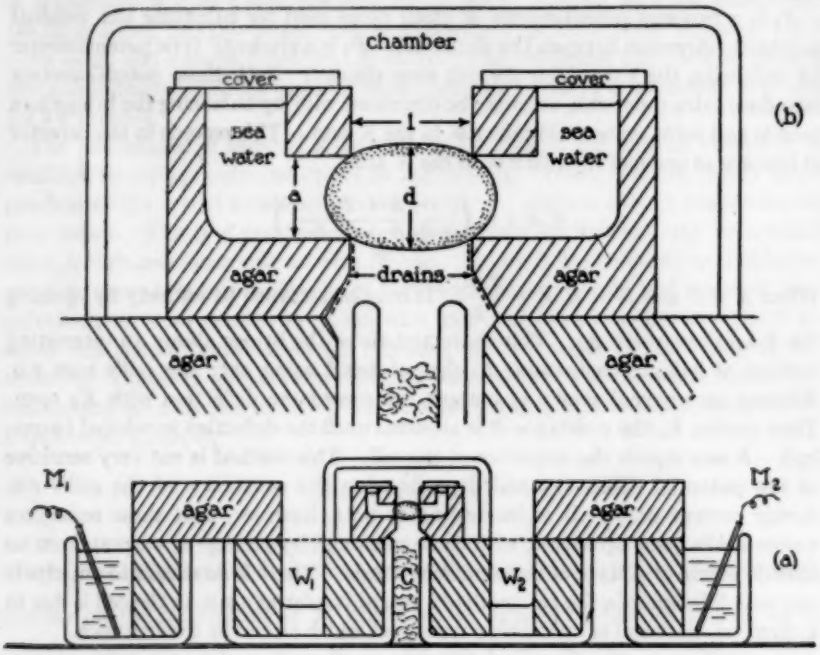

FIG. 3*a*. Apparatus used to hold *Valonia* cells for resistance measurements. FIG. 3*b*, enlarged view of agar blocks holding cell.

altering its permeability. Attempts to make a tight seal by means of vaseline or lanolin are fruitless, since the oil does not adhere to the wet wall and moisture soon creeps in, making conducting channels of uncontrollable size. The simplest and most uniform method is to hold the cell gently at the ends only and allow the cellulose wall itself to be the only electrical leak around the cell. Agar blocks, into which shallow convexities are hollowed, or into which holes are made at right angles by cork borers, hold the cell firmly yet with no injury for several hours if necessary. The agar is very stiff, so that the size and shape of the aperture is not

appreciably changed during the experiment. This is important in making a good check with the dead cell placed in the same position. The air gap (l) and the aperture (a) are carefully measured in each case with a micrometer caliper in order to replace cells accurately. When different solutions are desired in rapid succession they can be pipetted into the vertical cup and frequently renewed, so that diffusion from the agar does not markedly contaminate them. For greater surety other blocks saturated with the given solution may be substituted. In these experiments sea water only is used.

Removing and replacing the cell several times gives reasonably good checks, the steady values usually agreeing within 5 per cent when the dimensions are carefully adjusted. These are best, and the cell least injured, when it is protected from evaporation, but even in the open air cells have been in place several hours a day for many days without injury, the losses being made good by intake of water from the cups. A good seal with the latter is desirable although solution leaking from the contact is carried off by the filter paper strips laid on the agar as drains. Very little fluid is lost when the sea water wells are covered with agar or glass plates as shown in the figure.

In utilizing the apparatus a good cell, spherical or cylindroid in shape, and free of adhering growths, is rolled gently on filter paper and dried. The agar blocks are likewise freed from surplus moisture. The cell is placed in the left aperture, chosen to be slightly smaller in diameter than the cell, then the other similar block is moved up and gently pushed against the right end of the cell. If necessary, paraffin wedges are placed at the farther ends of the blocks to exert a slight pressure; but friction on the lower blocks is usually sufficient to maintain the cell in position. The wells are entirely filled and covered, and the filter paper drains brought just below the contact of the cell with the agar.

In making measurements, a small potential is applied (15 to 20 mv.) its value being noted on V. This is practically that applied to the cell itself since the resistance of vessels and blocks is very low (10 to 20 ohms or less than 1 per cent of that of the cell). The galvanometer, if deflected, is brought to zero by an adjustment of R. The potential is now increased slowly and the resistance adjusted again. The deflection is increased and it is possible to adjust with greater accuracy. With cells described above as "normal" a change from as low as 10 mv. to as high as 100 mv. produces no change of resistance. Above 100 mv. injury may occur which reduces the resistance, the reduction being greater the higher the potential. For "normal" cells below 100 mv., Ohm's law is followed quite accurately, the current being proportional to the potential and the resistance therefore constant. Neither is there any rectifying effect, the resistances being equal with the current flowing in either direction.

After several readings have been taken, the cell is removed and replaced in order to determine the error due to distance, dryness, etc. With the greatest care in duplicating conditions the scatter may be as great as 100 ohms in 2000; and it is difficult to obtain much better agreement. Some typical values are shown in Table I.

TABLE I

Direct Current Resistances of Valonia ventricosa

Dimensions of cells in cm.			Resistance in ohms		Dead resistance as per cent of live resistance
l	d	a	Live	Dead	
1.0	1.4	1.0	1600	28	1.7
1.2	1.3	1.0	1600	35	2.2
1.1	1.2	1.0	1690	32	1.9
3.0	3.0	2.6	1700	27	1.6
1.7	1.2	1.1	2050	53	2.6
2.1	2.2	1.7	2000	50	2.5
2.3	1.5	1.3	2300	40	1.7
2.5	1.75	1.1	2800	40	1.5
2.4	1.5	1.3	3000–3400	67	2.0–2.2
1.9	1.1	0.9	5000–5400	55	1.0–1.1

The dead resistances are determined by killing the cell with chloroform, heat, mechanical agitation or by high voltage. The resistance is seen to be invariably less than 100 ohms (usually from 20 to 40 ohms, depending on the dimensions of the cell). This value agrees with the specific resistance of the extracted sap which is approximately equal to that of 0.6 N KCl solution.

It is immediately evident that the living protoplasm interposes a considerably higher resistance than the dead. This has long been realized, but there are few cases where the difference between living and dead values is so great as in *Valonia*. In *Laminaria*, for example, there is a reduction on death to about 10 per cent of the living value. Here the reduction may be nearly to 1 per cent. The difference is probably due to the amount of leakage around the cell through the wall. In *Valonia* this is reduced to a minimum by the thin wall and its isolation for considerable distance in an air gap. It is doubtful if any arrangement short of actually naked protoplasm could better this condition.

It is necessary to determine this leakage in order that the resistance of the protoplasm may be evaluated. Two methods are used which we may designate as direct and relative. The direct method aims to compare the resistances of protoplasm with a virtual non-conductor such as air. Theoretically this should give absolute values, but practically the real value of the shunt through the wall is sufficiently uncertain so that all we can hope for is a lower limiting value. The relative method arrives at a value for protoplasmic resistance by exposing different areas of its surface to the flow of current and comparing the resistances. The conditions here can be made nearly independent of variability in the shunt and the findings have some added value, though they are not free from criticism.

In the direct method the cellulose wall is isolated and measured in conditions as nearly comparable as possible to the living state. The sap and protoplasm are removed through a fine capillary, and sea water is introduced several times into the cavity to wash out the remaining material. The wall is gently pressed dry on filter paper and inflated with air through the capillary. This wall is tough and flexible but does not stretch and when inflated returns to its original volume (as measured by displacement in water). It is put in position between the agar blocks and carefully pressed while being inflated with air so that the contacts and gap between the block are exactly as when the cell was alive; when the capillary is removed the cell remains expanded. The resistance is measured again. Ordinarily this first substitution, when carefully made, duplicates remarkably well the average of the live resistances. As the wall stands, however, even in a closed chamber, the imbibed water tends to evaporate, and as there is now no replacement of moisture from the protoplasm the resistance rises. If the cell is freshly drenched with sea water (taking care none enters the puncture) the course of the draining and drying may be followed by the resistance changes. These are shown in Fig. 4 for a number of successive drenchings and drainings. After the first 5 minutes the rise is slow but gradual, and is taken to mean drying, since no drops accumulate on the lower side. The value at 5 minutes is taken for comparison with the live cell at the same time. It appears that the dead wall has a slightly higher resistance generally, even at 5 minutes, than the live cell. Several factors may contribute, all being in the direction of an increase.

Irreversible changes take place in the wall if it is kept too long after the protoplasm is removed. Thus long drying appears to render it later incapable of taking up as much water—it becomes brittle and friable, and the resistance is high. Conversely, if the isolated cell wall is kept for some time in water it becomes more spongy and tends to separate more readily into laminae (perhaps due to bacterial action). It is not a new claim that the cell wall is markedly different in life and in death. We might expect greater changes the longer time the protoplasm has

been removed, and it may be significant that the values agreeing most closely with those of the living cell are obtained immediately after killing, on the first expulsion of sap and protoplasm. It seems possible that successive cleanings and dryings alter the thin layers of cellulose on the interior of the wall, these delicate parts being nearest the protoplasm and most recently laid down. (A laminated structure is easily demonstrated in the wall of *Valonia*.)

Another factor which would tend to make the live resistance appear lower than that of the wall alone is a possible conducting layer at the surface of

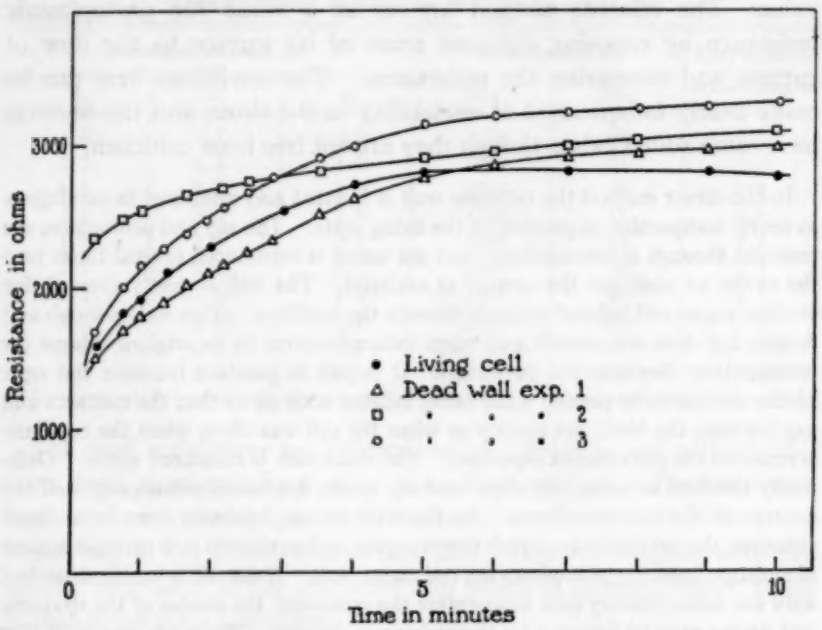

FIG. 4. Graphs showing course of several successive drainings and dryings of the same cell in the order indicated. There is some straightening of the curves at about 5 minutes; draining is now complete and the rise of resistance is now due to drying out.

the protoplasm. This might be non-living, yet be washed off when the protoplasm was removed. Or the protoplasm itself might offer a path for the current just inside the wall, while opposing its flow across the vacuole. The turgor of the cell might play a part in determining the thickness of this conducting layer, but its influence is not direct, since cells which have stood in the apparatus a long time, so as to become definitely soft, may have a higher resistance than when fresh or when replaced in slightly diluted sea water long enough for them to regain turgor.

Since all the changes which may occur, when the protoplasm is replaced by air, are in the direction of an increase of resistance it is evident we can only set a lower limit to the resistance of protoplasm. This can be calculated from the relation

$$2P = \frac{RW}{W - R}$$

where the resistance of each layer of protoplasm is P, that of the total live cell is R, and that of the wall is W in the simple shunt of Fig. 5 (S is negligible for these calculations). We determine W and R as described. In a cell of 1 cm. diameter, R is often about 2000 ohms. This may be uncertain to within 5 per cent, and W is consistently about 10 per cent higher, in other words, $R = 2000$, $W = 2200$. In this case we calculate $2P = 22{,}000 \pm 1000$. Knowing the area of the cell in contact with the cups we can calculate the surface resistance. In this case the area was close to 1 sq. cm. for each end, so that the value is 11,000 ± 500 ohms per square centimeter of surface. This may safely be taken as a lower limit, and there is every indication that it is much higher. Table II shows some extremes of calculated values.

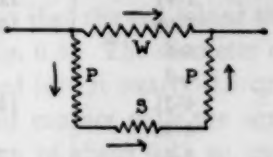

FIG. 5. Diagram of resistances in the cell. W, wall; P, protoplasm; S, sap.

Other factors will make the specific surface resistance still higher. Thus the current will tend to flow across the protoplasm not only at the surface of contact but for some distance along the cell as well, and the effective area will be larger by part of the surface in the air gap. Also there is evidence, to be presented in later papers, that by far the greater resistance is located at the point where the positive current enters the cell, and not where it leaves. Therefore $2P$ will more nearly give the single surface value. Since we have no indication of the thickness of the insulating layer in these cells, the calculation of specific resistance is not very significant. But taking 0.001 cm. for the total thickness of protoplasm, we arrive at a value of 10 megohms per centimeter cube, which is a very respectable insulator.

The relative method shows that the actual resistance of the protoplasmic surface may be even greater. This method makes use

TABLE II

D. C. Resistances of Valonia Cells by Direct Method

Cell No.	Live resistance R	Air-filled wall (5 min.) W	Calculated resistance of protoplasm $2P = \frac{WR}{W-R}$
	ohms	*ohms*	*ohms*
1–69	2700–3000	2800–3300	14,800–75,000
1–75	1100–1150	1130–1280	7,800–50,000
1–81	1350	1690–1710	6,550
1–84	1650–1670	1390–1500	∞
1–91	1300–1580	1380–1770	4,900–22,500
1–95	1400–1500	1730–1850	5,750–11,300
4–6	2000	2100	42,000
4–7	1600	1550–1600	∞
4–21	1400–1700	1700	7,900–∞
4–40	4300	3500–4700	50,000
4–42	1700–1800	1500–1800	30,500
4–57	1400–1540	1350	∞
4–67	1500–1600	1550	46,000

Note: The value of 2P = ∞ means that the living cell offered as much resistance as the air-filled wall, or more. The apparently greater live resistance must be due to insufficient removal of moisture from within the dead wall. Similarly very low values of 2P may be due to excessive drying of the air-filled wall, or in some cases to injury in the living cell. A good value of P is about 10,000 ohms.

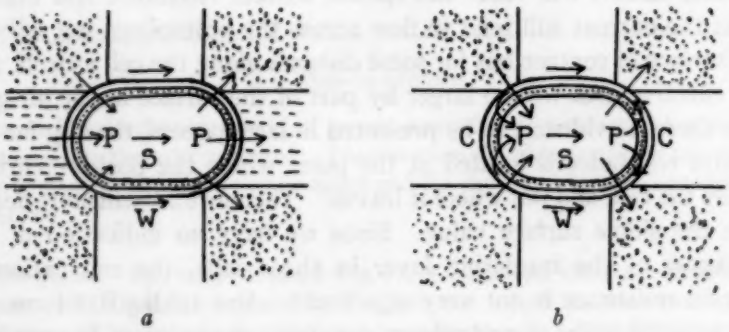

FIG. 6. Diagram of current paths with (*a*) full aqueous contacts, (*b*) narrow agar contacts. *W*, wall; *S*, sap; *P*, protoplasm; *C*, center of annular contact.

of the agar blocks shown in Fig. 6 *a* and *b*. Occasionally slow leaks at the area of contact with the agar drain the sea water from the wells; it had been observed in the course of the work that this made little difference in the resistance providing the cell was kept from drying out. In other words, when the area of contact was much reduced by lowering the level in the well, surprisingly enough the cell conducted as well as before. This might mean that the protoplasm was supplying little or no path for current *across* it. The experiment was repeated with the conditions more carefully controlled. The agar blocks were thoroughly dried of all surface moisture, and the cell likewise. Contact was made very carefully so that the cell joined the agar in a very narrow annulus only, as in Fig. 6 *b*. The diameter of the opening was 1 cm., and the cell projected into it nearly 0.5 cm. with a hemispherical surface. The width of contact with the agar was about 1 mm., making an annular contact of about 0.15 sq. cm. The resistance, soon reaching a steady value, was 1570 ohms ± 5 ohms. The wells were now filled with sea water and covered, no leaks developing. The resistance was not distinguishably changed—less than 0.5 per cent. Yet the area of contact was increased from 0.15 sq. cm. to more than 1.5 sq. cm., a tenfold change. Geometrically the resistance of the protoplasm should have changed inversely. Thus if the surface resistance at full aqueous contact were $\frac{11{,}000}{1.5} =$ 7350 ohms, at $\frac{1}{10}$ contact it should be 73,500 ohms and the resistance of the cell rise from 1570 ohms with full contact to 1945 ohms with $\frac{1}{10}$ contact. As a matter of fact the expected change will be smaller if we allow for the spread of current away from the contacts, either in the cell wall or in adhering films of sea water. This is indicated in Fig. 6 *b*. It will tend to widen the effective contact with the protoplasm and make the changes much less. Under the extreme conditions of a thick wall and well conducting protoplasm, the difference might be nearly abolished between Fig. 6 *b* and 6 *a*. But we know the resistance of the wall in this case to be such that over this area a resistance of 500 ohms exists between the annular contact and the center *C*. This would be equal to an average series resistance between contact and protoplasm of 250 ohms. *P* would equal 7300 + 250 = 7550 ohms and the change from full to partial contact would change the

value of cell resistance from 1555 to 1570 ohms—a very slight but detectable difference. A series of readings taken with full and partial contacts failed to give any consistent differences with "normal" cells, while with the "variable" cells, where we know that much more current passes the protoplasm, there was a slightly lower resistance with the full contact than with the partial.

This technique is more sensitive with long narrow cells where the leakage along the wall is lower and the total resistance is higher. Hence it is better adapted to *Nitella* with walls imbibed with tap water than to *Valonia*. Experiments of this kind with *Nitella* will be described in a later paper. For the present purpose this method gives further assurance that the resistance of the protoplasm is very much higher than that of the cell wall shunting it.

V

The theoretical import of the high resistance of *Valonia* to direct current depends on the assumptions made as to the physical nature of the protoplasm. The simplest of these is that the surface is a poorly conducting film, possibly lipoid in nature, which acts like the dielectric of a condenser in its admittance to alternating current, but has the conductivity of an imperfect insulator. A small current would then be carried by the movement of ions already present, or entering from sap and sea water. These would be presumably of both charges. If, as in the case of calcite,[9] or glass,[10] ions of only one charge are carried, the non-aqueous phase should act as an electrode, reversible to the ions which can pass, and giving potentials dependent on their concentration. This is the principle of the glass electrode, and the evidence for *Valonia* indicates that it, too, is the seat of potential differences dependent on the solutions with which it is in contact.[3,6] These P.D.'s could be produced either in a system of liquids with phase boundary potentials, or at a solid surface such as an electrode. The high resistance in the former would then be due to the low concentration or the low mobility of ions in the non-aqueous phase. The cell, however, is the seat of polarizations, and we have not been able to

[9] Joffé, A. F., The physics of crystals, New York, 1928, p. 114.
[10] Quittner, F., *Ann. Physik*, (4), 1928, **85**, 745.

observe such phenomena of any magnitude in such a phase boundary system as amyl alcohol in contact with aqueous solutions of LiCl and KCl. We must, therefore, turn elsewhere for analogies.

High resistances at an electrode surface may be due to the presence of films (*cf.* the aluminum anode, which has many suggestive similarities to the protoplasmic behavior) or to polarization. The latter takes place when by the flow of current a certain number of ions have been moved up to the electrode and concentrated,[11] producing a counter E.M.F. which decreases the effective E.M.F. through the circuit. Current ceases to flow almost entirely when the counter E.M.F. becomes equal and opposite to the applied one. Slight leakage, or continued conduction occurs for two reasons:

1. Substances concentrated at the electrode surfaces diffuse away slowly and decrease the counter E.M.F., allowing a few more ions to move up. This is the "residual current" or "Reststrom."

2. Among the ions moved up to the electrode there may be some which are discharged upon the electrode[12] and are not therefore accumulated in the solution. These are the ions to which the electrode is *reversible*. These may be in small amount compared to the total concentration of ions present. Thus an electrode in a mixed solution would not be polarized by currents small enough to discharge only ions to which it is reversible, but would become so when such ions were depleted by the passage of large currents. This can be seen with calomel or hydrogen electrodes, and is suggested by *Valonia* cells in the variable state. The interpretation of the latter in terms of ionic reversibility has been partly worked out, and will be discussed elsewhere.

The complete interpretation of the high resistance of *Valonia* to direct current in the normal state must be deferred to later papers giving other electrical evidence such as the time relations made evident in alternating current and "transient" D.C. measurements. The present data show that little or no ionic interchange takes place across the protoplasm even though there are gradients favorable to it. The cells are normally exposed to very different concentrations of ions on opposite sides of the protoplasm, and the latter is the seat of very appreciable potential differences.[6] The direct current measurements

[11] Either as ions or as discharged melecules dissolved in the solution.

[12] *I.e.*, incorporated into the metal, or second phase.

are thus an experimental verification of the slight movement of ions occurring under such conditions.

Most of these studies were carried out at the Laboratory of Marine Biology, Dry Tortugas, Florida. It is a pleasure to acknowledge the courtesy and cooperation of the Carnegie Institution of Washington in offering its facilities during the past four summers.

SUMMARY

A direct current bridge with vacuum tube detector is described for measuring the resistance of living cells. Methods for evaluating the surface of contact with the protoplasm, and the leakage around the cell wall, allow us to calculate the effective resistance of the protoplasm. In *Valonia ventricosa* this is usually at least 10,000 ohms per square centimeter and is often much higher. This is in agreement with the very slight ionic interchange observed in normal *Valonia*.

THE DARK ADAPTATION OF THE EYE OF LIMULUS, AS MANIFESTED BY ITS ELECTRIC RESPONSE TO ILLUMINATION

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In a previous paper (Hartline, 1928) there has been reported the result of a study on the electric action potential of the arthropod eye, with respect to the dependence of its form and magnitude upon the stimulating light. It is well known that the electrical responses of photoreceptors to illumination also depend upon a number of other factors which are not concerned with the stimulus, but which exert their influence upon the condition of the photoreceptor tissue; such factors are temperature, adaptation, oxygen supply, etc. It is the purpose of this paper to deal with one of these factors, namely, the condition of adaptation of the eye, and in particular to follow the course of dark adaptation as manifested by the behavior of the action potential.

Material and Method

The electric response to a light-stimulus manifests itself by a change in the potential difference existing between the corneal surface and the back of the eye. The form of these electric responses, and the apparatus and methods used in obtaining and recording them have been fully described (*loc. cit.*).† With the introduction of the following

* National Research Fellow in the Medical Sciences, 1927–1929.

† Briefly, the method is as follows: The animal is securely fixed, by means of pins and plaster of paris, in a light-proof moist-chamber. A water-immersion microscope objective focuses a very small image of the filament of an electric lamp upon the cornea of the animal's eye. The droplet of immersion fluid is balanced isotonic saline (*i.e.* ordinary seawater in the present experiments), and furnishes contact for one electrode. The other electrode makes contact on the

modifications the method used is identical with that described in the paper cited.

1. The electrical measuring system has been improved by a vacuum tube circuit in connection with the string galvanometer. Vacuum tube circuits are now so generally used in physiological technique that details of apparatus need not be described, excepting to state that the grid bias was sufficient to render the system completely potential-operated, thus eliminating polarization effects at the electrodes and changes due to fluctuation in resistance of the preparation. The entire unit was calibrated carefully to insure linearity of deflection with impressed potential.

2. The single brief flash of stimulating light was obtained by a rotating-disk shutter. The apparatus consists of a disk from which is cut a sector of known angle, rotating in front of a slit placed in the path of the light on its way to the eye. The duration of each flash is determined by the angular aperture of the sector and the speed of revolution of the disk. The disk is driven by a motor whose speed is regulated to any desired value, within 1 per cent, by means of a stroboscopic method. An automatically operated electro-magnetic shutter serves to single out one flash of the succession set up by the rotating disk. The operation of this shutter is accomplished by means of a commutator, cut in the form of a coarse screw in a bakelite drum, and mounted on the shaft carrying the disk. A flexible steel wire, with a platinum point, engages the screw, and when released is carried over the metal contact embedded in the groove of the screw. Contact is made over only part of a revolution, and the magnetic shutter is thereby activated, the disk and commutator being properly adjusted so that only a single flash of light is allowed to pass. The tip of the steel wire is carried on to the end of the screw, and must be re-set before another exposure can be made. This device made it possible to obtain a range of exposures from 0.0005 to 0.4 second.

The material employed in this study was the compound eye of young *Limulus polyphemus*. The action potential of this photoreceptor has been described in the previous paper (*loc. cit.*), and was seen to possess a simplicity of form which other arthropod eyes, as well as vertebrate retinas, do not show. The response to illumination is a simple wave of potential, rising sharply to a maximum, and subsiding more slowly toward the original base-line. It shows no other element, regardless of whether the illumination is continued or is only a momentary flash. In addition to this simplicity of form, the *Limulus* eye,

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animal's body, near the eye. These electrodes are connected through the necessary accessory apparatus, to an Einthoven galvanometer, and the electric changes arising in the eye are recorded photographically.

in the intact animal preparation,* survives and gives consistent results over a period of many days; this is indispensible in the study of such a slow process as dark adaptation.

RESULTS

The recovery of sensitivity, in the dark, by a photoreceptor which has been subjected to prolonged illumination is familiar to all; it is termed dark adaptation, and the course of the process has been investigated quantitatively by many experimenters. The criteria which have been used in detecting this recovery of sensitivity are many and diverse; the electric response furnishes yet another.

Upon being returned to darkness after an exposure of several minutes to a bright light, the eye of *Limulus* shows only a feeble response in the first minute or two, but as time goes on its response to the same stimulus becomes greater and greater, until it finally reaches the maximum value it can have for that stimulus. The exact course of the process is shown in Fig. 1.

In this experiment the eye was exposed to the brightest available light (*ca.* 85,000 meter candles) for 10 minutes; this light was then turned off, and at successive intervals thereafter the eye was stimulated by a flash of the same light, 0.002 second in duration, and of constant intensity. The magnitude of the maximum of the ensuing potential wave was measured, and is plotted as ordinate against time as abscissa. It is seen that the response elicited by a stimulus of constant energy increases rapidly during the first few minutes in the dark, and then more slowly, approaching a limiting value asymptotically.

The precautions observed in making these experiments are not unusual; the temperature must be carefully controlled, the intensity and duration of the stimulating flash must be regulated properly. The initial illumination must be carefully controlled as to its brightness; its exact duration, however, is not so important, as the eye is very nearly completely light-adapted in 10 minutes. Preliminary experiment has shown that the stimulating flashes themselves have little effect upon the condition of adaptation of the eye, consequently

* The intact-animal preparation has been used throughout this study; excised eyes give strictly comparable though less consistent results, and last only a few hours.

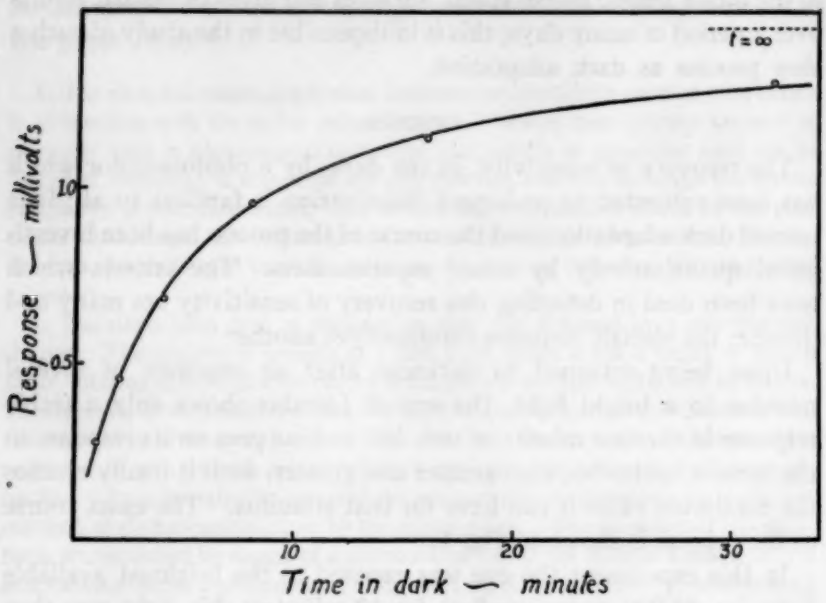

FIG. 1. The course of dark adaptation in the eye of *Limulus*. Ordinates: Magnitude of maximum of potential wave ensuing from stimulation by light. Abscissae: time in darkness. Initial adapting illumination, *ca.* 85,000 meter candles; area of eye illuminated, *ca.* 0.1 mm.^{2}; duration of initial illumination, 10 minutes; cessation of this initial illumination marks the instant $t = 0$. Stimulating flashes of same brightness as initial illumination; duration of stimulating flashes, 0.002 second.

Circles are experimental points; smooth curve is theoretical, given by equation

$$kt = \frac{1}{E_\infty - E} + C$$

where E_∞ is mean of two experimental values, obtained after *ca.* 2 hours in darkness k and C are obtained graphically from a plot of experimental values of $\frac{1}{E_\infty - E}$ against t.

The experimental points are measurements of individual action potentials, obtained from the same preparation within a period of about 3 hours.

I have four additional experiments similar to this, from three other animals, and numerous other less extensive experiments which are all fitted, to within the limits of experimental error, by the same type of theoretical curve.

three or four points on the curve, particularly if they are widely spaced in time, may be determined after each period of initial illumination.

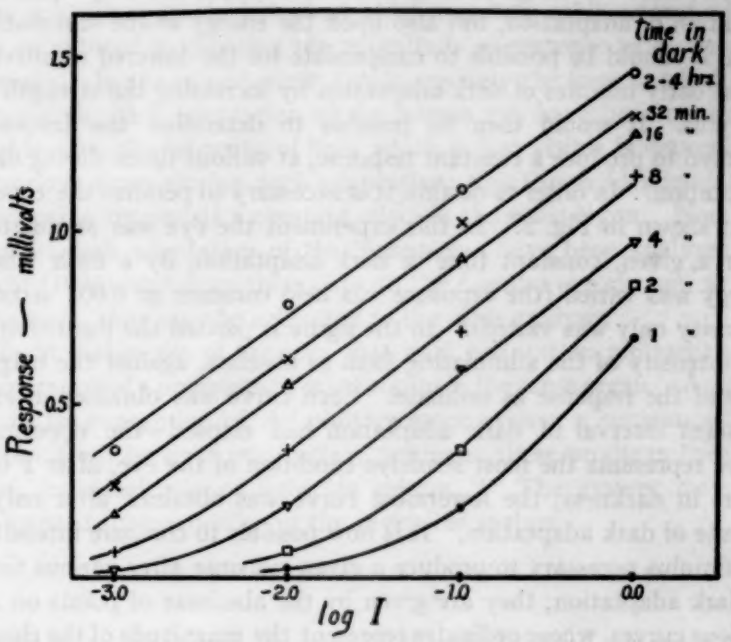

FIG. 2. Relation between intensity of stimulus and magnitude of response, at various times during dark adaptation. Ordinates: Magnitude of potential wave ensuing from stimulation by light. Abscissae: logarithm (base 10) of intensity of stimulating flashes. Duration of flashes = 0.002 second. Each curve obtained after a time t had elapsed, during which the eye was in darkness, subsequent to an initial illumination, as in Fig. 1. Values of t belonging to each curve given at the right. Intensity corresponding to log I = 0.0, *ca.* 85,000 meter candles. Area illuminated *ca.* 0.1 mm.^{2}

The experimental points are measurements of individual action potentials, obtained from the same preparation within a period of about 15 hours.

Intensity of stimulating light was controlled by the use of photometrically calibrated Wratten "Neutral Tint" filters, as described in the previous paper.

This is the only experiment of its type which I have performed.

Two or three such "runs" thus serve to determine satisfactorily the curve of the process. Repetition of such an experiment, particularly

if made within 10 or 12 hours, yields results identical to within 3 to 4 per cent.

Now, since the magnitude of response depends not only upon the condition of adaptation, but also upon the energy of the stimulating flash, it should be possible to compensate for the lowered sensitivity of the early minutes of dark-adaptation by increasing the strength of stimulus. It would then be possible to determine the intensity required to produce a constant response, at various times during dark adaptation. In order to do this, it is necessary to perform the experiment shown in Fig. 2. In this experiment the eye was stimulated, after a given, constant time of dark adaptation, by a flash whose energy was varied (the exposure was held constant at 0.002 second; intensity only was varied). In the figure is plotted the logarithm of the intensity of the stimulating flash as abscissa, against the magnitude of the response as ordinate. Each curve was obtained after a constant interval of dark adaptation had elapsed—the uppermost curve represents the most sensitive condition of the eye, after 2 to 4 hours in darkness; the lowermost curve was obtained after only 1 minute of dark adaptation. It is now possible to compare intensities of stimulus necessary to produce a given response after various times of dark adaptation; they are given by the abscissae of points on the various curves, whose ordinates represent the magnitude of the chosen response.

The first point of importance to be noted is that it will make no difference what value is chosen for the required response, the experiment shows that (within the limits of the method and material) all members of this family of curves have been obtained from a single curve by a shift along the axis of abscissae. It is with the amount of this shift—the same for all ordinates—that we are concerned.*

The reason for wishing to obtain the data in this form is two-fold. In the first place, it is an experimental fact, readily verified, that two records of action potential, obtained under different conditions of

* This would be even more evident had the uppermost curves in this particular experiment been carried further, using still lower intensities. The tailing off of the lower portion of the curve is always present; for the completely dark adapted eye it has been abundantly figured in the previous paper (*loc. cit.*).

stimulation and adaptation, but rising to the same maximum value, may be superimposed, and found to agree throughout their entire length, except for chance minor variations.* The data in their present form are consequently independent of a certain arbitrariness which was introduced in choosing the magnitude of response as a criterion of study. In the second place, this is precisely the form in which the data on the dark adaptation of the human eye have been obtained. In this case, the intensity of light which is just visible is determined at various times during dark adaptation; this threshold stimulation presumably represents a constant effect in the sense organ. Now the data on dark adaptation of the human eye have been analysed by Hecht (1920), and since these data on the *Limulus* eye are now in the same form, they may be subjected to the same analysis.

Hecht makes use of the idea that dark adaptation represents the regeneration of a photosensitive substance in the sense organ. According to his conception of the photoreceptor system a substance S is broken down by light into certain products; these products then reunite, independently of light, to reform S. The system may be represented schematically by the reversible system

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A \qquad (1)$$

The initial exposure to a bright light decomposes most of S, with the consequent accumulation of the products of its decomposition, P and A. When the light is turned off, the recombination of P and A is no longer opposed by photolysis, and S is regenerated, with the consequence that the sensitivity, which depends on the concentration of S, increases from a low value in early dark adaptation to its original value, when S is entirely regenerated.

This scheme has been developed and used by Hecht in an extensive series of papers; the application of it to the process of dark adaptation in several different animal forms is to be found in a recent paper

* I have investigated this only for the responses greater than 0.1 mv.—it is possible that the smaller responses—especially those obtained in the first few seconds of dark adaptation—deviate from this finding. It is furthermore necessary to compare only curves obtained at the same temperature.

(Hecht, 1927); the present treatment, however, is taken directly from his earlier paper dealing with the processes in the human eye (*loc. cit.*).

To account for the data quantitatively, it is necessary to assume a relation between the concentration of S and the intensity of light necessary to produce a given response. This assumption, which is by no means entirely arbitrary, is that the concentration of S is given by a linear function of the logarithm of the intensity. Since the condition of complete dark adaptation, when 100 per cent of S is present, requires the smallest intensity, the assumption becomes

$$[S] \propto -\log I \tag{2}$$

Choosing some one ordinate—say 0.5 mv.—the abscissa of the point on the 2 hour curve represents 100 per cent of S; the amount of S at any time, t, during dark adaptation is then represented by the abscissa of the point having this same ordinate, on the curve corresponding to t. The difference between the two abscissae represents—and, since the plot is logarithmic, is proportional to—the amount of S which is absent at that time, or, what is the same thing, the amount of products of decomposition which are present. On the basis of Hecht's assumption, then, the distance that a given curve has been shifted to the right from the 2 hour curve may be taken proportional to the concentration of products of photolysis which are combining to regenerate the photosensitive substance. A plot of this distance against time represents the course of the reaction, and would be expected to follow the known laws governing chemical kinetics. This indeed appears to be the case, and, as in the human eye and the photoreceptors of several lower animals, the order of the reaction is two; that is, the rate of recombination is proportional to the second power of the concentration of the combining substances. It is for this reason that Hecht has written two members on the right hand side of his schema (1).

The integrated form of the bimolecular isotherm is

$$kt = \frac{1}{x} + C \tag{3}$$

where t is the time of dark adaptation; x is the concentration of P (and of A); C a constant of integration, and k is a constant of proportionality.

On page 387, Vol. 13, No. 3, January 20, 1930, the numbers in the last column of Table 1 should be divided by 10 to give the true values of k'.

In the next to the last line, under Table 1, for *value of* $C = 0.337$ read *value of* $C = -0.337$.

In the case of the present data, x is to be calculated from (2) according to the equation

$$x = m(\log I - \log I_\infty)_E, \qquad E = \text{const.}, = 0.5 \text{ mv.} \tag{4}$$

where I is the intensity required to produce the response E at the time t, I_∞ is the intensity required to produce the same response when $t = \infty$ (2 hours), and m is a factor of proportionality which according

TABLE 1

Intensities of Stimulus Necessary to Produce a Response of 0.5 mv, at Various Times During Dark Adaptation

t (minutes)	$\log_{10} I$	$\log_{10} I - \log_{10} I_\infty$	$\frac{1}{\log_{10} I - \log_{10} I_\infty}$	k'
∞ (2–4 hours)	$\bar{3}.290$	—	—	
32	$\bar{3}.625$	0.335	2.98	0.83
16	$\bar{3}.870$	0.580	1.72	0.87
8	$\bar{2}.362$	1.072	0.93	0.74
4	$\bar{2}.778$	1.488	0.67	0.85
2	$\bar{1}.280$	1.990	0.50	0.82
1	$\bar{1}.670$	2.380	0.42	0.83

First column: time in darkness (*cf.* Fig. 2). Second column: abscissae ($\log_{10} I$) of points on various curves of Fig. 2, having an ordinate = 0.5 mv. Third column: $\log I - \log I_\infty \left(= \frac{x}{m} \text{ (cf. text)} \right)$. Fourth column: $\frac{1}{\log I - \log I_\infty}$. Fifth column: k' (*cf.* text), calculated according to equation

$$k' t = \frac{1}{\log I - \log I_\infty} + C.$$

Value of $C = 0.337$ obtained by graphic extrapolation from a plot of $\frac{1}{\log I - \log I_\infty}$ against t.

to the assumption is a constant, and whose value is determined by the units in which concentration is measured. When (4) is substituted in (3) it is seen that it is possible to calculate a new factor $k' = mk$, the constancy of which is a test of the applicability of the bimolecular isotherm to the present data. Table 1 gives the calculations involved. It is evident that k' is, within the limits of the method, a constant.

An additional fact is worthy of notice. It is seen that the upper

parts of the curves of Fig. 2 are linear, and, as has been remarked, quite closely parallel. It is consequently clear that, in making the analysis of the data, one could just as well have taken the difference in the ordinates of points having the same abscissa as the difference in abscissae of points having the same ordinate. This follows from a simple proposition of geometry, that the direction in which a straight line cuts a family of parallel straight lines does not affect the ratio of the segments. This procedure just mentioned merely changes the scale, *i.e.*, the units in which concentration is measured. Equation (4) becomes

$$x = m'(E_\infty - E)_I, \qquad I = \text{const.} \tag{5}$$

where m' is different from the m of (4).

In the light of this experimental fact the original assumption becomes much simpler, *viz.*, that the magnitude of electrical response, *provided it be sufficiently large*, is a linear function of the concentration of S. Indeed, Hecht's assumption originally was based upon the fact that the photochemical effect in photoreceptors is, in general, proportional to log I—which is an experimental fact also for the larger electric responses. It may seem, then, that the treatment given has been rather indirect; it must be clearly understood, however, that this simpler alternative is subject to the limitation that small responses, where the response is no longer linear with log I, must not be included, whereas the first treatment given is general.

This experiment which measures the ordinates at the same abscissa is much simpler to perform than the other; it is, indeed, the experiment of Fig. 1. Here the difference between the value of the response after prolonged dark adaptation, and that of the response at any time t, is proportional to the concentration of products of photolysis. The curve itself represents the course of the dark adaptation reaction, the points being experimental values of response, the smooth curve being theoretical, calculated according to (5) and (3).

The constancy of k', in Table I, and the close fit of the curve to the experimental points in Fig. 1, are results that would be predicted according to Hecht's conception of the photosensory process, a conception which was reached by fundamentally different methods from that employed in this study. This close agreement may be taken as

indicating that the electrical responses of the eye to illumination are intimately related to the fundamental photoreceptor process, and, indeed, constitute a sensitive measure of the decomposition and regeneration of the photosensory substance.

SUMMARY

1. The phenomenon of dark adaptation of the eye of *Limulus* is reflected in the behavior of the action potentials obtained upon stimulation by light. The method of obtaining and recording these action potentials has been described in an earlier paper.

2. By determining the intensity of stimulus necessary to produce an electric response of a given magnitude (as to maximum action potential), at various times during dark adaptation, a quantitative analysis of the process may be made. This analysis is identical with that of Hecht for the dark adaptation of the human eye.

3. The results of this analysis indicate that the process of dark adaptation in the *Limulus* eye may be represented by a chemical reaction of the second order—the recombination of products of photolysis to renew the depleted supply of photosensitive material. This is in complete accord with Hecht's conception of the photosensory process, and is in quantitative agreement with the results obtained by other methods, in several different animal forms.

4. The experimental relation between strength of stimulus and magnitude of electric response reduces the assumption originally made by Hecht to account for the data on the human eye to an equivalent form; that the magnitude of electric response, provided it be sufficiently large, is directly proportional to the concentration of the photosensitive material in the sense organ.

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SPECTROPHOTOMETRIC STUDIES OF PENETRATION OF DYES

VII. Dyes Absorbed by a Non-Medullated Nerve

By MARIAN IRWIN

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, December 30, 1929)

I

INTRODUCTION

Previous experiments[1] have indicated the danger of drawing conclusions as to the nature of a dye taken up by living cells without properly identifying it by means of spectrophotometric analysis. For example, methylene blue solution generally contains as an impurity a lower homologue, azure B, which to the eye appears rather like methylene blue but which gives a different absorption curve. When living cells of *Nitella* or *Valonia* are placed in such a solution, the color of the dye in the external solution, in the cellulose wall, and in the vacuole appears somewhat similar to the eye, but the spectrophotometer shows that while the dye taken up by the cellulose wall is identical with that in the external solution, giving an absorption curve characteristic of methylene blue, the dye which has penetrated the vacuole consists predominantly of azure B.

An application of the spectrophotometric method seems likewise desirable in connection with the absorption of dye by living nerve, the staining[2] of which has been heretofore investigated without making such tests. For this reason data are presented here on absorption of methylene blue, azure B, and acid fuchsin by the living non-medullated nerve of a lobster claw.

[1] Irwin, M., *J. Gen. Physiol.*, 1926–27, **10**, 927; 1928–29, **12**, 147, 407; *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 425.

[2] For review of literature see Lee, A. B., The microtomist's vade-mecum, eighth ed., P. Blakiston's Son and Co., Philadelphia, 1921; and Krause, R., Enzyklopädie der Microskop Technik, third ed., vols. 2 and 3, Urban and Schwarzenberg, Berlin, 1926.

II

Method

The lobster claw was removed from the body, and the nerve was exposed sufficiently to allow the dye (dissolved in sea water) to bathe it for a few minutes. That the treatment did not seriously affect the irritability of the nerve was shown by a vigorous response of the muscles when the end of the stained nerve was pinched by a pair of forceps. The nerve was invariably tested in this manner and was then removed from the claw and rinsed for a second in a large volume of sea water. The nerve was examined under a binocular microscope and in case connective tissue was present it was removed by means of fine forceps. After the dye solution on the surface was removed by rinsing in sea water the nerves were placed in a small volume of sea water (the number of nerves depending on the extent of staining) until there was enough dye extracted for spectrophotometric analysis. Three successive extractions were made during a period of 8 hours and the measurements were made immediately after each extraction.

For acid fuchsin it was necessary to stain the nerve in the sea water at pH 5.5 and to extract the dye also at this pH value, on account of the decolorization of the dye at the pH value of the sea water.

The extracted dye was shaken up with air to avoid error arising from possible decolorization of the dye through reduction.

After the third extraction so little dye was left in the nerve that the extraction was discontinued to avoid possibility of errors arising from dealing with nerve which had stood in sea water too long after excision.

The spectrophotometric measurements were made by W. C. Holmes[3] with a Bausch and Lomb spectrophotometer (improved model), at the Marine Biological Laboratory in Woods Hole. I wish to thank Mr. Holmes for his collaboration and the Laboratory for its hospitality during the summer of 1927.

III

RESULTS AND CONCLUSIONS

Spectrophotometric measurements of the dye extracted by the sea water from the nerve of the lobster claw, previously stained in methylene blue, azure B, or acid fuchsin, show that the absorption curves of the extracted dye solution are identical with those of the dye solution in which the nerve was stained (Table I). Since the absorption curves for each dye are identical and characteristic they are not

[3] W. C. Holmes, Cooperating expert in the field of spectroscopy of dyes for International Critical Constants.

given in the text. In Table I, however, there are given the primary absorption maxima obtained from these curves, which determine the nature of these dyes more accurately than other parts of the absorption curves.

Three successive extractions of each dye within a period of 8 hours give the same results, showing that during this period there is no change in the nature of the extracted dye.

TABLE I

Spectrophotometric Measurements of Dyes Extracted from the Nerve of a Lobster Claw Previously Stained Intact

Dyes	Primary absorption maxima
	mμ
Methylene blue	
a. dissolved in sea water	665
b. first extraction from the stained nerve	665
c. second " " " " "	665
d. third " " " " "	665
Azure B	
a. dissolved in sea water	650
b. first extraction from the stained nerve	650
c. second " " " " "	650
d. third " " " " "	650
Acid fuchsin	
a. dissolved in sea water	545
b. first extraction from the stained nerve	545
c. second " " " " "	545
d. third " " " " "	545

By this procedure most of the dye was removed so that the total extraction represents the predominant dye absorbed. But if, as in *Valonia*, a small amount of another dye, present as impurity in the external solution, is taken up by selective absorption it would not be detected by this method. When from an intact cell of *Valonia* which has been stained in methylene blue solution we extract the dye as in the case of nerve by allowing it to stand in sea water, the azure B which has collected in the vacuole often escapes detection. This is

because the concentration of azure B diffusing out of the vacuole is too small in proportion to the amount of methylene blue diffusing from the cellulose wall. Such a small amount of azure B in the vacuole may be detected, however, if the vacuole is punctured by a glass capillary tube and the sap containing the dye is extracted.

Unfortunately it is not possible to collect the dye from any portion of the nerve in such a manner. Nor is it possible to remove every bit of the dye from the nerve without possibility of error arising from continuing the extraction too long after excision. Furthermore, observing the staining at the cut end of the nerve does not help us since it does not necessarily represent the condition of the intact nerve. The only knowledge we can obtain regarding the penetration is that the dye absorbed by the nerve consists chiefly of the dye to which the nerve is exposed.

SUMMARY

Spectrophotometric measurements show that the non-medullated nerve of a lobster claw is capable of absorbing methylene blue, azure B, or acid fuchsin dissolved in sea water but this does not necessarily imply that the dyes penetrate into the interior of the nerve.

THE NON-LOGARITHMIC ORDER OF DEATH OF SOME BACTERIA

By OTTO RAHN

(From the Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca)

(Accepted for publication, December 20, 1929)

In a previous paper, the author (1929) has calculated the order of death for organisms which are killed by the reaction of just one molecule in the cell, and also for organisms which require the reaction of 2, 3, 4 and more molecules before the cell is injured beyond recovery. It was shown that higher organisms follow the order of death as calculated for several reacting molecules, while bacteria, as a rule, follow the order computed for one reacting molecule per cell.

This can be best illustrated graphically by plotting the logarithms of the survivors on a standard time scale where 100 units represent the time necessary to kill 99.9 percent of the initial number of organisms. Fig. 1 shows a straight line when the reaction of one certain molecule in the organism causes its death; all other cases show a different type of curve, bulging out above the straight line.

It has been further shown that the death rate of organisms computed from the formula

$$0.434\ K = \frac{1}{t} \log \frac{a}{b}$$

(where a is the initial number of organisms, and b the number of survivors after the time t) is constant for *one* reacting molecule, but increases steadily if more than one molecule reacts.

While bacteria often show a constant death rate and a straight line survivor curve, it happens frequently that the death rate decreases and the survivor curve sags below the straight line (see dotted line in Fig. 1). Both these deviations indicate that this cannot be caused by more than one molecule reacting because the effect would have been just the opposite.

As early as 1908, Miss Chick accounted for this by the assumption of a graded resistance. The death rate of any inhomogeneous mixture of bacteria will be high at first, because the less resistant forms die rapidly. Towards the end of the experiment, the weak individuals are practically all dead, and the death rate is determined by the more resistant cells. Each grade follows the logarithmic order, but the

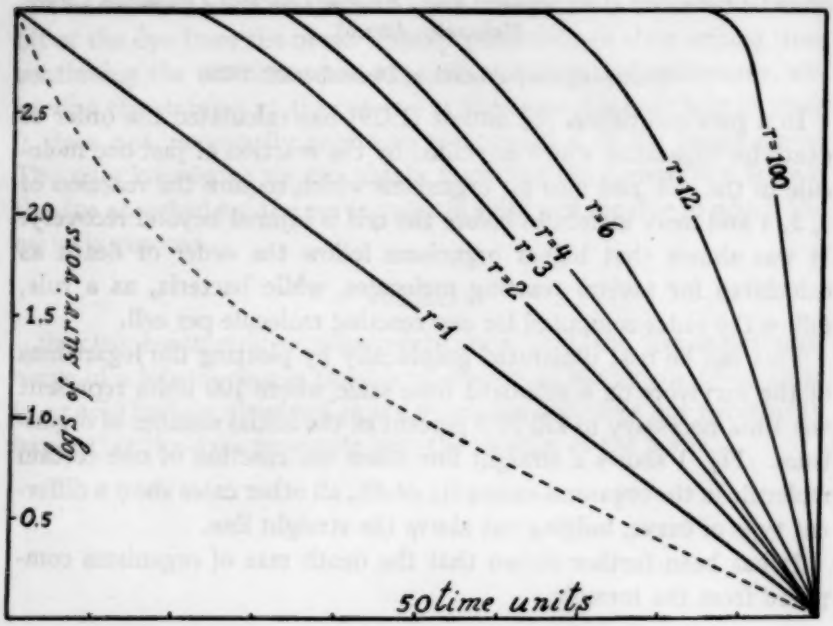

FIG. 1. Theoretical survivor curves, if 1, 2, 3, 4, 6, 12 or 100 molecules must be inactivated to kill the cell. Dotted line: Survivor curve of a mixture of bacteria of different resistance.

sum of all of them results in a sagging curve and a decreasing death rate, as can be seen from Table I, where the survivors from three strains of different resistance have been calculated, each having a constant death rate, while the death rate of the sum of survivors is seen to decrease distinctly. The logarithms of the total survivors give the dotted line of Fig. 1.

Falk and Winslow (1926) tried to account for this type of death

order by assuming multimolecular rather than monomolecular reactions. As long as there is no absolute proof of a graded variability of resistance in bacteria, this kind of explanation must be considered as one of several possibilities. The general type of survivor curve would correspond to the dotted line of Fig. 1. But it seems to the author that the assumption of a varied resistance is much more probable.

A very interesting experiment confirming the author's view is the gradual change of the order of death in Reichenbach's experiments

TABLE I

A Theoretical Case of Disinfection with Three Grades of Resistance and r = 1 Reacting Molecule Per Cell

Time	Survivors in each group			Survivors total	Death rate	Standard time units
	$K = 1.0$	$K = 0.2$	$K = 0.05$			
0	100,000	800,000	100,000	1,000,000	0	0
1	10,000	504,000	89,130	603,130	0.220	2.5
2	1,000	318,500	79,440	398,940	0.205	5.0
3	100	201,000	70,800	271,900	0.188	7.5
4	10	126,800	63,100	189,910	0.180	10.0
5	1	80,000	56,240	136,241	0.173	12.5
10	0	8,000	31,630	39,630	0.140	25.0
15	0	800	17,790	18,590	0.115	37.5
20	0	80	10,000	10,080	0.100	50.0
25	0	8	5,624	5,632	0.090	62.5
40	0	0	1,000	1,000	0.075	100.0

(1911) with cultures of *Bact. paratyphosum* of different age. A direct comparison is difficult, because the average rates differ greatly, and the initial number of cells varies also. But by expressing the survivors in percents of the initial number, we have the same starting point of 100 percent. For graphic comparison, it becomes necessary to standardize the time as well. This was accomplished by calculating the time necessary to kill 99.9 percent of all cells. According to the formula for the death rate

$$0.434\ K \cdot t = \log \frac{a}{b}$$

we find

$$0.434\ K \cdot t = \log \frac{100}{0.1} = 3$$

or

$$t = \frac{3}{0.434\ K}$$

TABLE II

Order of Death by Heat of Bact. paratyphosum with Cultures of Different Age, on Standard Scale

5½ hours old			8 hours old			13 hours old			18.5 hours old		
Heated to 49°			Heated to 48°			Heated to 50.1°			Heated to 51°		
Time units	Survivors	0.434 K	Time units	Survivors	0.434 K	Time units	Survivors	0.434 K	Time units	Survivors	0.434 K
0	100	—	0	100	—	0	100	—	0	100	—
7.1	3.185	0.748	1.7	70.5	0.074	3.0	90.5	0.022	9.1	44.8	0.174
17.8	1.417	0.355	4.4	26.8	0.114	7.5	57.7	0.047	22.8	19.93	0.140
35.7	0.607	0.222	8.7	10.4	0.098	15.1	32.8	0.048	45.6	6.54	0.119
53.6	0.265	0.166	13.0	5.52	0.084	22.8	15.3	0.054	68.4	0.88	0.137
71.4	0.224	0.133	17.4	3.63	0.072	30.1	7.84	0.055	91.3	0.186	0.137
89.3	0.131	0.113	21.8	2.40	0.068	37.6	4.87	0.052	100.0	0.100	0.137
100.0	0.100	0.107	26.1	0.845	0.069	45.1	3.40	0.048			
			34.8	0.745	0.053	60.2	1.33	0.049			
			43.5	0.646	0.044	75.3	0.48	0.046			
			52.2	0.596	0.037	90.3	0.205	0.045			
			65.2	0.745	0.028	100.0	0.100	0.046			
			78.2	0.497	0.026						
			100.0	0.100	0.026						

24 hours old			28 hours old			48 hours old			55 hours old		
Heated to 50°			Heated to 51°			Heated to 49°			Heated to 50°		
Time units	Survivors	0.434 K	Time units	Survivors	0.434 K	Time units	Survivors	0.434 K	Time units	Survivors	0.434 K
0	100	—	0	100	—	0	100	—	0	100	—
4.8	88.4	0.021	12.3	46.65	0.165	10.8	40.1	0.080	4.3	74.9	0.063
11.9	71.7	0.027	30.9	13.68	0.176	21.7	18.17	0.074	10.8	45.5	0.068
23.8	46.8	0.032	61.8	1.388	0.186	32.6	11.24	0.063	21.7	17.7	0.076
35.7	24.85	0.037	92.6	0.214	0.178	43.5	4.07	0.069	32.6	6.90	0.077
47.6	12.20	0.045	100.0	0.100	0.192	54.3	2.83	0.062	43.5	3.07	0.076
59.5	6.025	0.048				65.1	1.55	0.060	54.3	1.41	0.074
71.5	1.630	0.059				87.0	0.331	0.062	65.2	0.915	0.068
95.2	0.116	0.071				100.0	0.100	0.065	82.0	0.249	0.065
100.0	0.100	0.072							100.0	0.100	0.065

This time might also be determined graphically. We consider it as standard, and call it 100 units, and compute the minutes of the experiment into these units. Reichenbach's data, computed in this way, give the standardized values of Table II.

In Fig. 2, the logarithms of the percents of survivors are plotted against standard time. The youngest culture shows the greatest deviation from the straight line of logarithmic order, indicating a very

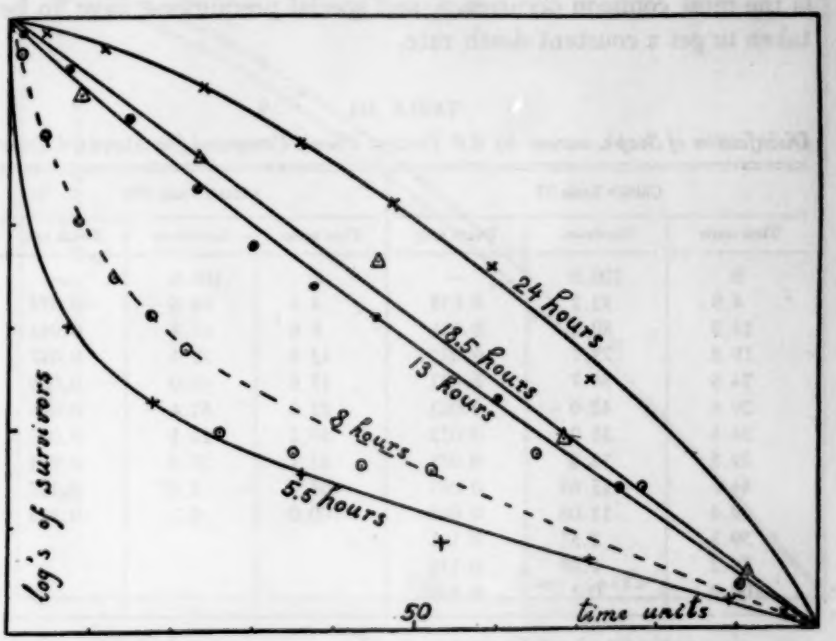

FIG. 2. Survivor curves of *Bact. paratyphosum* cultures of different age, drawn to standard scale.

inhomogeneous mixture of quite resistant (old) and quite sensitive (young) cells. With increasing age, the rapid multiplication eliminates, absolutely and relatively, the resistant cells. The 8-hour culture was not carried far enough to kill 99.9 percent of all cells. Several extrapolations are possible, and this curve in Fig. 2 is therefore dotted. At 13 hours, the cells are fairly homogeneous. At 24 hours, the survivor curve goes beyond the straight line. Reichenbach considered this one experiment abnormal but he could not account

for the deviation, and consequently published it with the others. Another experiment with a 24-hour old culture gave normal results. The old cultures are very near the straight logarithmic order. This one exception shows an increasing death rate while the death rate of the others is either constant, or decreasing. The greater the decrease, the deeper is the sagging of the survivor curve.

The decrease of the death rate is nothing uncommon. Really, it is the most common occurrence, and special precautions have to be taken to get a constant death rate.

TABLE III

Disinfection of Staph. aureus by 0.6 Percent Phenol Computed for Standard Scale

Chick's Table VI			Chick's Table VII		
Time units	Survivors	Death rate	Time units	Survivors	Death rate
0	100.0	—	0	100.0	—
4.9	92.2	0.035	4.3	84.6	0.073
14.9	80.8	0.031	8.6	81.8	0.043
19.8	73.7	0.033	12.9	72.4	0.047
24.8	54.7	0.052	17.2	63.0	0.050
29.6	42.0	0.063	22.0	52.4	0.056
34.6	31.0	0.073	30.2	26.2	0.083
39.5	18.8	0.091	43.1	10.4	0.098
44.5	15.65	0.089	64.7	2.47	0.107
49.4	12.08	0.092	100.0	0.1	0.115
59.3	3.51	0.121			
74.2	1.69	0.118			
100.0	0.1	0.149			

Quite rare, however, is the increase of the death rate with bacteria, while it is the common occurrence with higher organisms. The most remarkable thing about the few cases of increased death rates recorded in literature is that they are characteristic for certain species of bacteria. The first typical case was observed by Chick (1910) with Staphylococci. It is very striking that one single glance at the survivor curves in Miss Chick's paper will tell at once whether it represents a staphylococcus or any of the other bacteria tested. The curves of the Staphylococci resemble the survivor curves plotted by the author for the assumption that 2, 3, 4 or more molecules in the cell have to be reacted upon in order to cause death (see Fig. 1).

The increasing death rate and the shape of the curves are characteristic for death by heat as well as for death by phenol. This fact, together with the uniformity of the logarithmic order of death by all kinds of different agents such as heat, drying, freezing, light, chemical poisons, make it quite probable that the same molecules are affected in each case. The previous paper had been limited to death by heat,

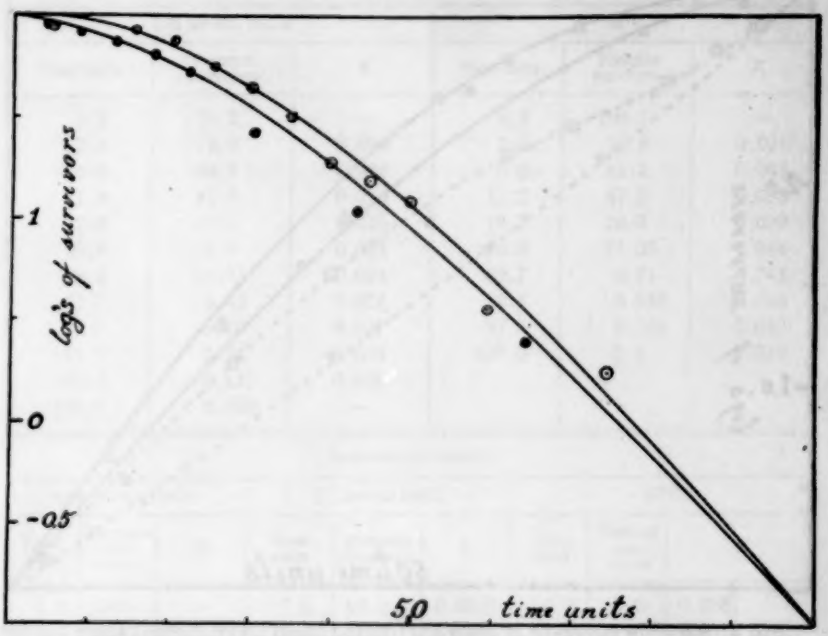

FIG. 3. Survivor curves of *Staph. aureus*, drawn to standard scale.

but from now on, death by chemical poisons will also be considered though the homogeneity of the system has not been proved.

Two tables of Chick's on *Staph. aureus* are represented in Table III as computed on the standard scale used before. In her other tables, the initial number of bacteria which shows the most important criterion for this type of curves, namely, the eventual period of no deaths or retarded death is missing; without this, the other data are of no value for our purpose.

A slow rate of dying is observed from the very beginning, in Chick's

experiments as well as in the others to be discussed later, but this might be accounted for by variation in resistance. A computation of the curve for various grades of resistance with more than one molecule reacting gives results similar to those shown in Fig. 3, and the following curves.[1]

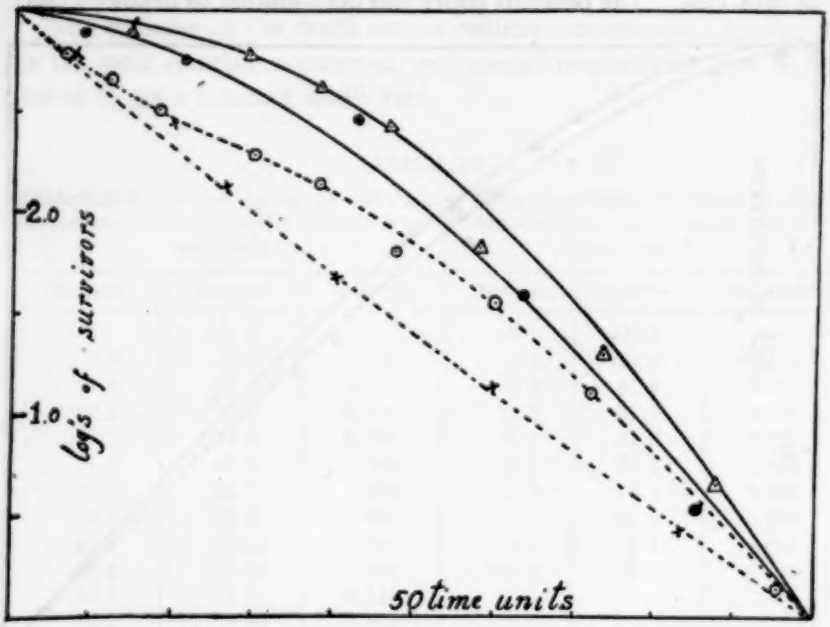

FIG. 4. Survivor curves of *B. anthracis* (dotted lines) and unnamed spore-former (full lines) drawn to standard scale.

Another typical case of increased death rate is recorded by Reichenbach (1911). This investigator found that the death rate with spores of *B. anthracis* was fairly constant whether heat or bichloride of mercury was used, while with the spores of an unidentified bacillus, the death rate increased whether heat or bichloride of mercury was used. The essential fact again is the observation that the order of

[1] After the completion of the manuscript, the paper of Gates (1929) appeared in which a culture of *Staph. aureus* gives a survivor curve very similar to the one shown in Fig. 3.

death is not a function of the killing agent, but a property of the organism.

TABLE IV

Order of Death of Spores of Two Bacilli

B. anthracis

0.05 percent $HgCl_2$			87°C.		
Time units	Percents survivors	K	Time units	Percents survivors	K
1.2	76.2	—	1.3	100.5	—
2.4	74.9	0.013	2.6	90.8	0.021
6.0	60.7	0.026	6.6	61.2	0.043
11.9	45.0	0.026	13.2	47.2	0.033
17.9	33.1	0.026	19.7	26.0	0.039
29.9	19.0	0.025	26.5	13.05	0.044
38.2	13.73	0.024	39.7	4.71	0.044
47.8	6.42	0.028	59.5	0.852	0.046
59.7	3.76	0.027	83.3	0.268	0.040
71.7	1.34	0.030	100.0	0.1	0.039
95.6	0.137	0.035			
100.0	0.100	—			

Unnamed spore-former

0.05 percent $HgCl_2$			0.1 percent $HgCl_2$			87°C.			
Time units	Percents survivors	K	Time units	Percents survivors	K	Time units	Percents survivors	K	
1.5	88.5	—	7.4	95.2	0.0109	4.2	85.6	0.068	
2.9	86.3	0.0105	18.4	42.7	0.0739	8.5	75.6	0.061	0.058
7.3	84.3	0.0052	36.9	20.1	0.0697	21.2	56.6	0.050	
14.5	77.2	0.0066	55.4	5.86	0.0822	42.4	29.58	0.053	
21.8	67.5	0.0084	73.8	0.787	0.1070	63.6	3.79	0.095	
29.1	59.2	0.0092	92.2	0.244	0.1056	84.8	0.322	0.125	
37.8	41.7	0.0130	100.0	0.100	0.1160	100.0	0.100	0.131	
46.6	26.3	0.0170							
58.2	6.54	0.0290							
72.8	1.97	0.0337							
87.3	0.46	0.0387							
100.0	0.10	0.0424							

Reichenbach's data were computed to standard scale (Table IV) and are shown graphically in Fig. 4. *B. anthracis* approximates a

straight line, while the unknown spore-former behaves distinctly different and bulges out above the straight line.

In 1912/13, Eijkman resumed his studies of the order of death. He investigated especially the influence of the size of organism on the shape of the survivor curve and found that "the assumption seems justified that while the smaller microorganisms behave analogous to the monomolecular reaction, with the larger organisms, the individual

FIG. 5. Types of survivor curves observed by Eijkman.

TABLE V

Death of Spores of Bacillus 25 in Alkali

pH 13.12			pH 11.4			pH 7.5		
Time units	Log's of survivors	K	Time units	Log's of survivors	K	Time units	Log's of survivors	K
0	3.00	—	0	3.00	—	0	3.00	—
13.6	2.87	0.075	20.5	2.62	0.0073	14.8	2.75	0.00058
27.3	2.70	0.086	41.0	2.12	0.0084	29.6	2.50	0.00058
54.6	2.18	0.118	61.5	1.52	0.0095	44.3	2.20	0.00061
82.0	1.02	0.190	82.0	0.76	0.0108	59.2	1.82	0.00068
100.0	0.00	0.252	100.0	0.00	0.0118	74.0	1.38	0.00075
						88.8	0.77	0.00086
						100.0	0.00	0.00108

differences in resistance might become more conspicuous and might express themselves in the survivor curve."

He distinguished 4 types of curves which are presented in Fig. 5. Type A was found in the death of small spores (*B. subtilis*) and medium sized spores (*B. anthracis*). Type B in some experiments with *Bact. coli*, Type C in two experiments with *Bact. coli*, and always with pink yeast and bread yeasts. Type D was found only once, with the spores of a very large, unnamed bacillus.

Another set of data has been published which shows a non-logarithmic order of death with an unnamed spore-former, namely, those of Myers (1929) on the disinfection by strong alkalies. All experi-

ments but one showed an increasing death rate. It would be impossible to present all data here, but a random selection was made presenting one table each of the death by pH 7.5, pH 11.4, and pH 13.1. Since Myers mentions logarithms only for his averages, Table V also gives only the logarithms of the percentage of survivors. The survivor curves resemble those of Reichenbach's unnamed spore-former so much that they need not be presented here.

These three organisms, the staphylococcus and the two spore-formers, are the only bacteria which have shown an increasing death rate as a rule. Besides them, we have the yeasts, according to Eijkman, but they will not be considered here because they are not bacteria. The above types of survivor curves are not without exceptions, however. Lee and Gilbert (1917) report a very constant death rate with *Staph. aureus* killed by phenol. Among the 18 sets of experiments of Myers', one is quite constant and two others nearly so.

On the other hand, those species ordinarily following the logarithmic order show occasional exceptions. Reichenbach's one exceptional case with *Bact. paratyphosum* has been already mentioned. Then there are the two experiments of Eijkman's with *Bact. coli* showing plainly an increasing death rate, while two other experiments with the same organism gave a decreasing rate.

We might consider the evidence given above regarding the three organisms as an indication that they contain 2 or 3 "reacting molecules" per cell. But the considerable number of exceptions makes another explanation more probable. All three exceptional species will probably not give accurate plate counts. The Staphylococci ordinarily stick together in lumps or clusters, while the spore-formers are likely to grow in threads, each thread consisting of several cells containing one spore each. The clusters and threads will not be broken up into individual cells during the diluting and plating process, and each cluster or thread will give only 1 colony, regardless of the number of individual cells in it. A cluster of 8 cells will then count only as 1 cell, and after 4 of these cells are dead, it still counts as 1. Even after 7 of the 8 are dead, it will appear on the survivor curve as 1 cell. It is evident that such a cluster behaves exactly like a cell with 8 reacting molecules. If such clusters are in abundance, the survivor

curve of such inseparable organisms must be similar to that for several reacting molecules. This is identical with an increasing death rate.

Breed and Stocking (1920) compared the plate count with the direct microscopic count and found that with *Bact. coli* in milk, each colony on a plate corresponded to 1.2–1.6 cells in the average, while Whiting (1923) counting separately clusters and cells in milk, found that in the great average one clump of cells is represented with *Strept. lactis* by 2.8 cells, with other streptococci by 26.8 cells, with micrococci by 12.3 cells, with rods by 5.8 cells and with yeasts by 5.5 cells. For pure cultures of *Strept. lactis*, Baker, Brew and Conn (1919) found that each colony of the plate count corresponds to approximately 1.8 cells.

A true logarithmic order of death can be expected only when the plate count actually represents the number of single cells still capable of multiplication.

Some recent investigations with yeast in this laboratory have shown that a "bulging" survivor curve is found when the yeast is killed by temperatures very near the maximum temperature. Probably we are dealing here with repair processes which, at the very slow rate of death, might for a while be capable of keeping cells alive. An analysis of this type of curve will be given as soon as the experiments have been completed.

SUMMARY

In a previous paper, it has been shown that the logarithmic order of death of bacteria can be accounted for by the assumption that each cell contains one or several extremely sensitive molecules and that the destruction or inactivation of any one of these prevents multiplication of the cell.

In this paper, the apparent exceptions to the logarithmic order are dealt with. It has been shown that the *decreasing* death rate can well be accounted for by the assumption of a variation in resistance of the cells under test.

The few cases of *increasing* death rates might be indicative of a different cell structure, requiring the destruction of 2 or 3 molecules before multiplication is made impossible. More probable, however, is the assumption that these bacteria behave exactly like the others,

and that the apparently larger number of molecules per cell is caused by our imperfect method of counting living bacteria by the plating method where a cluster of several cells can be counted only as one cell. Bacteria with a tendency for clustering are likely to give results resembling the expectation for several reacting molecules per cell.

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THE PREPARATION OF A GRADED SERIES OF ULTRAFILTERS AND MEASUREMENT OF THEIR PORE SIZES

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The use of ultrafiltration as a tool in the investigation of bacteriological problems has become extremely common of late years. Perhaps the chief application of the process lies in measuring the particle size of ultramicroscopic viruses, and in reviewing the current literature one is struck by the gross disagreements of the estimates so obtained. Yet the method is basically simple. Theoretically, a suspension of the infectious agent whose particle size it is desired to ascertain is placed above a membrane of known pore size. Positive or negative pressure is applied, the filtrate is collected, and its infectiousness determined experimentally. One then can say the virus has passed through pores of such a size, it is therefore smaller than these pores; or the membrane has retained the virus, consequently, the latter is larger than the pore diameter.

However, while the concept of a sieve-like action of membranes is theoretically tenable, there enter into the process of ultrafiltration many factors which make it necessary to use the utmost caution in the interpretation of results. For example, the suspended agent may be adsorbed at the membrane surface; proteins in the dispersion medium may coat the pores thereby reducing the effective pore size of the membrane (Hitchcock (1)); the infectious agent may exist in a disperse phase of varying particle size depending upon the menstruum in which it is suspended (Krueger and Tamada (2)); the virus may undergo considerable dilution during filtration with the consequence that the filtrate is non-infectious; surface tension, pH and pressure conditions of filtration influence the course of the process, and there are, in short, innumerable factors involved all operating to render the data obtained

of questionable value unless the experimental conditions are rigorously controlled. The extreme discrepancies then between the results of various investigators are readily understandable in light of the complexity of the procedure and the fact that most ultrafiltration experiments have not been adequately controlled.

In the course of work begun some 2 years ago and for the most part already published (Krueger and Tamada (2) and Krueger and Schultz (3)) it became necessary for us to have available a system of ultrafilters of graded pore sizes. After considerable experimentation with some of the graded series reported in the literature we fixed upon that described by Bechhold (4) as best suited to our needs and modified his system in several details *q.v.i.* Elford (5) has also found the Bechhold series well adapted to bacteriological work and has reported most interesting results in a recent publication.

The present paper deals with our observations on acetic collodion membranes and is published with the hope that the collected data may be of value to those using ultrafilters for particle size determinations.

Apparatus and Preparation of Membranes

(*a*) *Apparatus.*—Fig. 1 illustrates the type of apparatus we have found to be satisfactory. The two halves are wrapped separately and autoclaved. Sterile heavy rubber washers which reduce the effective filtration area to 4 cm.^{2} are mounted on either side of the membrane to be employed, and the clamp placed in position. The material to be filtered is pipetted into the upper cylinder and the the latter is closed by inverting over it half a sterile Petri dish. Negative pressure is applied through the gooseneck tube (A), the filtrate collecting in the lowest part of the lower cylinder from which samples may be secured at any time by opening outlet (B). Two stopcocks are provided so that samples may be taken without disturbing the course of the filtration. It will be noted that the filtrate outlet is kept free from outside contamination by placing it in a sterile tube during the periods between sampling.

(*b*) *Preparation of Membranes.*—The membranes are prepared by impregnating Whatman No. 1 filter paper discs with acetic collodion solutions of various concentrations and gelling the collodion in water. Anthony's negative cotton is dried in a desiccator to constant weight and solutions of the following collodion content are prepared in glacial acetic acid: 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 per cent. These solutions are best made in quantity and kept in large bottles, removing the amounts needed for current work from time to time. The solutions for immediate use are placed in squat, covered cylindrical containers sufficiently

large to receive the filter paper. The surface of the solution is scraped free from bubbles and a disc of filter paper is lowered into it, taking every precaution to avoid bringing in air bubbles with the paper. When the paper has become thoroughly impregnated with collodion it is lifted up above the surface and slowly rotated in its own plane until the excess solution had completely run off. Flat broad-billed cover glass forceps are very helpful in this procedure. The disc is now

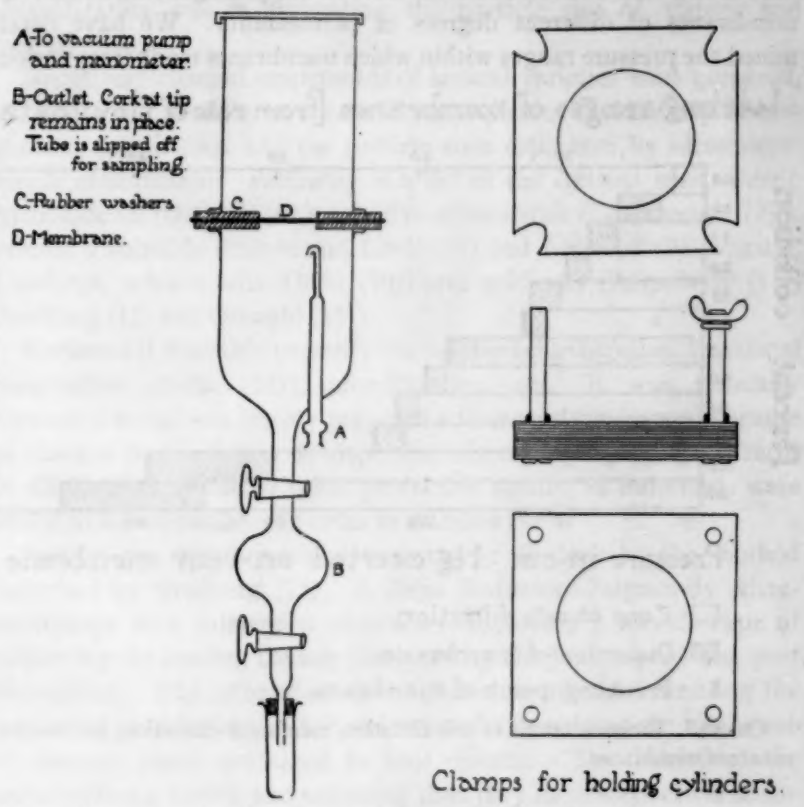

FIG. 1. Apparatus for ultrafiltration under aseptic conditions.

plunged into sterile distilled water again avoiding bubbles and is washed in successive changes until the wash water is free from acetic acid. Membranes prepared in this way can be stored in sterile water saturated with chloroform, for 2 weeks without changing in permeability. No special precautions for sterilizing the membranes are necessary since the original acetic collodion solutions are sterile and if aseptic technic is followed during the washing contamination will not occur. It has been our practice to test one membrane of each batch for sterility.

Properties of the Membranes

(*a*) *Pressure Ranges.*—In work with viruses and bacteriophage the negative pressures used were always small (not more than 10 cm. of Hg). Larger pressures resulted in marked distortion of the membranes and introduced a source of error in comparing filtration through membranes of different degrees of permeability. We have determined the pressure ranges within which membranes made from various

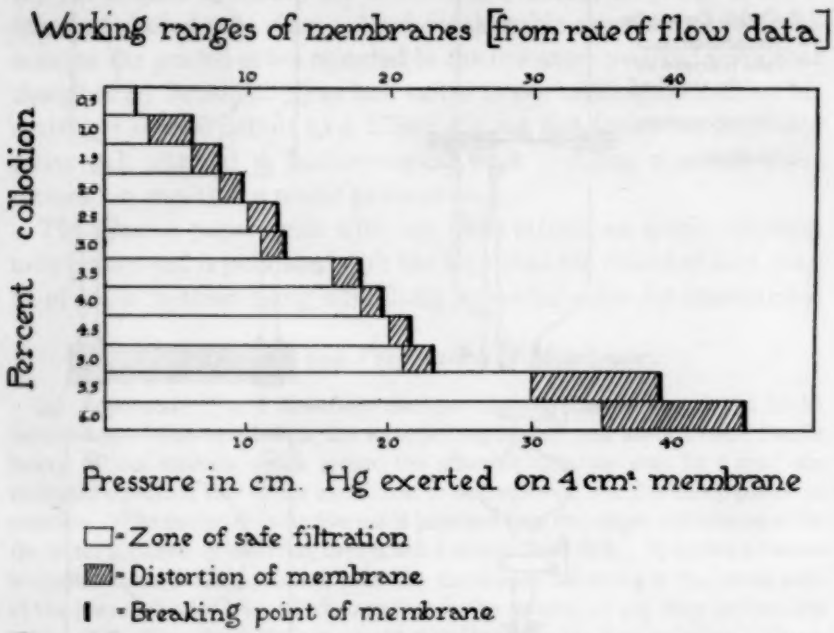

CHART I. Pressure ranges of safe filtration, membrane distortion, and breaking points of membranes.

percentages of acetic collodion may be used without danger of breaking or of developing gross leaks due to confluence of pores. Chart I indicates these values.

(*b*) *Permeability.—1. Permeability to Colloids of Known Size.*—Our interest lay not so much in determining the actual pore size of the individual membranes as it did in ascertaining the size of particles which would traverse the membranes. These are quite different values for any particular membrane and it is a common experience of

individuals working with ultrafilters to find that pores whose sizes are estimated from rate of flow data appear to be very much larger than the diameter of the largest particles for which the membrane is permeable. We graded our membranes then on the basis of permeability to colloidal particles of known size, since our chief use for ultrafiltration was in estimating the particle size of viruses and bacteriophage.

Negatively charged suspensoids of several varieties were prepared, purified when possible to obtain disperse phases of relatively homogeneous constitution and the particle sizes estimated by ultramicroscopic examination. Following is a list of the colloids used: Ferric hydroxide sol (Ostwald (6)), negative silver iodide (Lottermoser (7)), arsenic trisulphide (Picton and Linder (8) and Börjeson (9)), mastic, Gamboge, sulphur sols (Odén (10)) and gold sols (Zsigmondy (11), Svedberg (12) and Ostwald (13)).

We found it desirable to purify the sols by centrifugation, fractional coagulation (Odén (14)), ultrafiltration, etc. It was generally necessary to use sols freshly prepared and recently measured because of changes in the degree of dispersion accompanying aging. Traces of alkali, gum arabic or other protective agents, as indicated, were added to the suspensions in order to stabilize them.

Particle size determinations were nearly all done by the method described by Svedberg (15). A Zeiss Siedentopf-Zsigmondy ultramicroscope with immersion objectives employing a special type of rubber cup for holding the sols (designed by Dr. Jean Oliver) was used throughout. The estimations of sol diameters involved counting the number of particles in a certain volume and determining the total mass of disperse phase contained in that volume. The density of the particles being known and assuming that they have a spherical shape

$$r = \sqrt[3]{\frac{3\,M}{4\,\pi\,\rho\,n}}$$

where r = radius of particle, M = total mass of particles in given volume, n = number of particles observed in this volume and ρ = density of particles.

Frequently the disperse phase of a sol consisted of particles too small to be clearly seen in the ultramicroscope. In these cases the particles

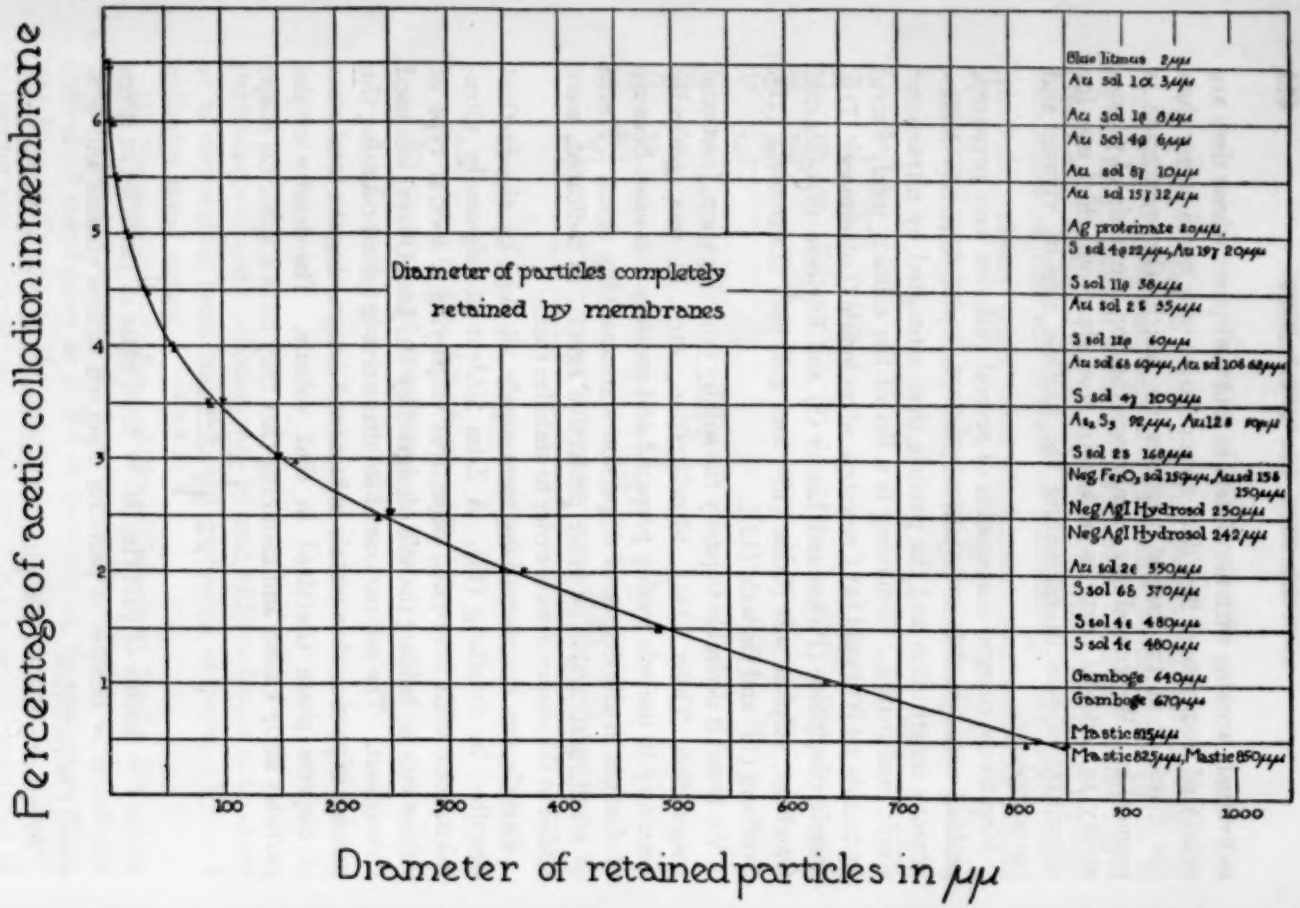
Percentage of acetic collodion in membrane
Diameter of particles completely retained by membranes
Diameter of retained particles in μμ
Blue litmus 2μμ
Au sol 1α 3μμ
Au sol 1β 6μμ
Au sol 4β 6μμ
Au sol 8γ 10μμ
Au sol 15γ 12μμ
Ag proteinate 20μμ
S sol 4β 22μμ, Au 19γ 20μμ
S sol 11β 38μμ
Au sol 2δ 35μμ
S sol 12β 60μμ
Au sol 6δ 69μμ, Au sol 10δ 82μμ
S sol 4γ 100μμ
Au2S3 92μμ, Au 12δ 89μμ
S sol 2δ 120μμ
Neg Fe2O3 sol 159μμ, Au sol 15δ 150μμ
Neg AgI Hydrosol 250μμ
Neg AgI Hydrosol 242μμ
Au sol 2ε 350μμ
S sol 6δ 370μμ
S sol 4ε 480μμ
S sol 4ε 480μμ
Gamboge 640μμ
Gamboge 670μμ
Mastic 815μμ
Mastic 825μμ, Mastic 850μμ

CHART II. Variation of diameter of retained suspensoid particles with percentage of acetic collodion in membrane.

were plated with gold following the methods of Zsigmondy (16), Westgren (17) and Börjeson (9) until they became large enough to be counted in the ultramicroscope. Knowing the mass of the nuclear particles the radii of the components of the original disperse phase could then be readily computed. The spontaneous formation of new nuclei was guarded against by controlling the conditions under which gold deposition occurred as suggested by Westgren (17). A further check lay in watching for deviations from the linear relationship existing between the volume of nuclear fluid used and the number of

TABLE I

Physical Properties of Membranes and Calculated Pore Radii

Percentage acetic collodion in membrane	Thickness l, in cm., $\times 10^3$	Gm. collodion/cm.^{2} m, $\times 10^3$	Gm. HOH/gm. collodion; e	Rate of flow c.g.s. units q, $\times 10^3$	Pore radius r, $\times 10^6$ from $r = l\sqrt{\frac{8\eta q}{(m)(e)}}$
0.5	21.3	0.31	46.8	900	47.48
1.0	21.9	0.42	38.2	580	37.23
1.5	23.6	0.49	33.1	245	25.96
2.0	24.2	0.58	32.0	210	23.03
2.5	25.0	1.31	18.1	172	19.05
3.0	25.9	1.58	15.9	108	13.98
3.5	28.0	1.83	15.0	66	12.26
4.0	29.7	1.90	14.2	39	10.11
4.5	30.1	2.26	14.2	30	8.25
5.0	37.5	2.52	14.0	19.9	7.99
5.5	48.8	3.01	13.6	10.2	6.88
6.0	67.2	3.83	12.5	5.8	6.60

particles in the product; a relationship holding only when no spontaneous development of particles is occurring.

In practice, three membranes of a certain grade were tested at one time. A sol of known particle size was filtered through each membrane under low pressures well within the ranges indicated in Chart I, and the sol concentration of the filtrate determined ultramicroscopically. Constantly larger particles were used until less than 0.1 per cent of the sol particles came through in the filtrate, when the membrane was classed as impervious to particles of that size. This arbitrary limit of 0.1 per cent was chosen to allow for deviations from the average particle size in a given suspensoid.

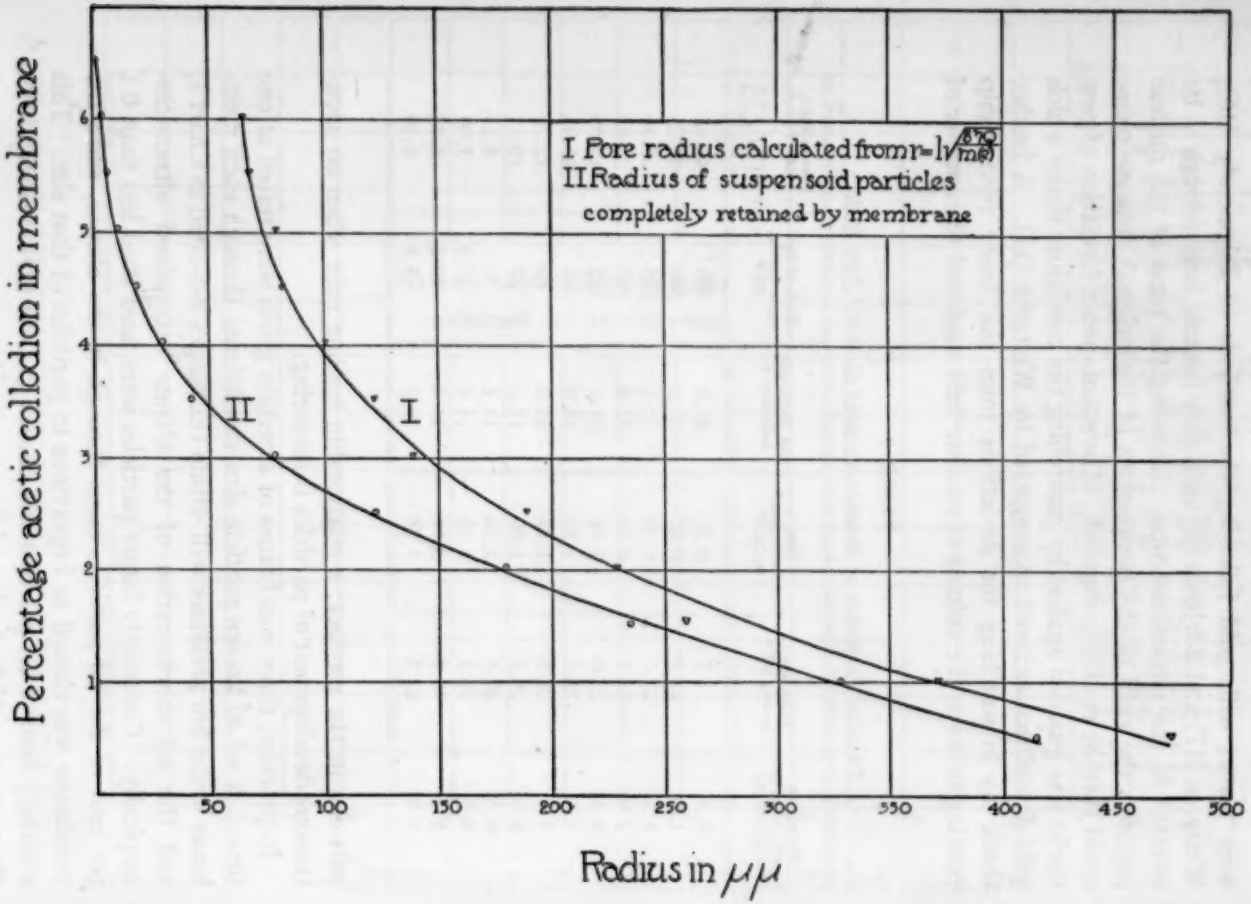

CHART III. Pore radii of membranes calculated from Hitchcock's formula compared with radii of suspensoid particles which each membrane completely retains.

Chart II represents graphically the permeability of the membrane series as measured by the procedure outlined above in several experiments with different batches of membranes. Opposite each ordinate value for percentage of acetic collodion occurs the diameter in $\mu\mu$ of suspensoids completely retained by the membrane, and at the right side of the chart are tabulated the particular sols used. In each case the next lower member of the series permits passage of >10 per cent of the same sol.

2. Permeability to Water.—Ten membranes of each percentage of acetic collodion from 0.5 to 6.0 per cent were measured for: (a) permeability to water, (b) thickness, (c) weight of water per gram of collodion, (d) weight of collodion per square centimeter of membrane, (e) weight of water per square centimeter of membrane. Table I lists the average figures obtained.

Hitchcock (1) found for the membrane system he was using that

$$r = l\sqrt{\frac{8\,\eta\,q}{m\,(w-1)}}$$

where
r = pore radius in centimeters.
l = thickness in centimeters.
η = viscosity coefficient of water.
q = rate of flow in c.g.s. units.
m = grams collodion per square centimeter.
$(w\text{-}1)$ = grams water per gram collodion.

Substituting the corresponding values in Table I in Hitchcock's equation we obtain the curve shown in Chart III. With it for comparison is shown the curve of retained particles plotted in Chart II.

DISCUSSION

We have particularly avoided even mildly complicated procedures such as vacuum impregnation, etc., in making the membranes, with the hope that the ease of preparation and measurement would lead bacteriologists and biologists to employ the Bechhold series more widely than heretofore. The acetic collodion membranes are in Elford's opinion and our own, very well adapted to bacteriological requirements.

It is interesting that membranes made in the manner described in

this paper should possess constant physical properties. Yet, once having established a satisfactory technic we have had no trouble in reproducing the series at will; while measurements of thickness, rate of water flow, etc., have not varied sufficiently to furnish cause for alterations in the mode of preparation. Chart II is a good criterion of the constancy of the series since it is a graphic composite of many separate experiments.

The difference between the curves for pore size as measured by permeability of the membranes to water and to colloidal particles is hardly to be wondered at. In the first place, Hitchcock based his formula on data obtained from experiments with collodion membranes having no cellulose base as did ours. Again, the fundamental process of gelling in the two instances differs considerably as Elford (5) has already pointed out. We are unable at the present time to furnish any quantitative corrections which will bridge the gap between the two types of measurements although it should be pointed out that there exists a constant difference between the two curves plotted in Chart III. That is, the values obtained from

$$r = l\sqrt{\frac{8\eta q}{mc}}$$

equal the colloidal particle radii plus a constant. It is conceivable that part of this difference may be due to water coating the pore walls as an immobile layer, a theory for which there is some experimental basis.

SUMMARY

A simple procedure for making a series of membranes of graded pore sizes is presented with data for permeability of the membrane series to water and to colloidal sols of known particle sizes. A convenient ultrafiltration apparatus for operation under aseptic conditions is described.

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PHOTOTROPISM AND THE LIGHT-SENSITIVE SYSTEM OF PHYCOMYCES

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I

The light-sensitive sporangiophores of the fungus *Phycomyces* exhibit two types of photic response: simple acceleration of growth, and phototropic bending. The former, which may be called the *direct growth response* in distinction to the *phototropic growth response*, typically follows symmetrical illumination of the sporangiophore from above or from opposed sources of equal intensity. Phototropic curvature on the other hand results from unequal illumination on opposite sides, as from exposure to lateral light from one source.

The direct growth response has been used as an index of the excitation of the light-sensitive system of the sporangiophore (Blaauw, 1914; Tollenaar and Blaauw, 1921) and quantitatively as a measure of its changing sensitivity during dark-adaptation (Castle, 1928–29). The phototropic response also may be used in studying the characteristics of this system. Such a use does not necessarily involve a knowledge of the relation between the two modes of photic response. The phototropic response is under certain circumstances better suited than the direct growth response to indicate excitation, although not so under all circumstances. For instance, in the experiments to be described the exposure of a sporangiophore to unilateral stimulating illumination for more than a relatively brief period (0.6 second) led to the abeyance of the phototropic response. This state is that of so-called "phototropic indifference," the nature of which will be discussed elsewhere.

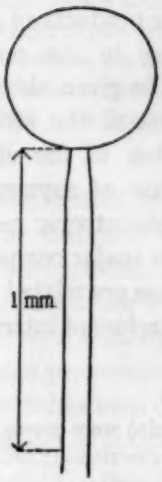
Aside from the use of the phototropic response as a tool, it is of interest in its own right as concerns the possible mechanism of phototropic

bending, and the way this bending may be related to the direct growth response. The eminently reasonable theory has been developed by Blaauw (1918), based on a variety of circumstantial evidence, that the phototropic bending of plants or their parts is due to unequal growth responses induced by unequal illumination. The direct growth response, called by Blaauw the *light-growth* response, is thus regarded as the primary agent in phototropism, and the curvature as therefore resulting from differential stimulation and response. By studying simultaneously and under comparable conditions the two modes of photic response shown by *Phycomyces* it should be possible to test the hypothesis that both are based on the same light-sensitive system. The test is carried out by showing that the reaction times as determined separately for each type of response vary similarly with a significant common variable, in this case the duration of the exposure to light (Castle, 1930).

Undoubtedly a number of steps or reactions in sequence intervene between the primary reception of light by a plant and the subsequent phototropic curvature. The number as well as the nature of these steps has been generally unknown, however, and failure to recognize the complexity which processes purely secondary to the photochemical action of light might introduce has led to doubt as to the validity of the simple theory of Blaauw. The subsidiary, non-photochemical events may even involve the transportation of growth-accelerating substances to a place of action remote from the locus of photic excitation, as in the shoots of *Avena* (Went, 1928). Obviously, if the measured index of the phototropic response is separated by a number of intervening processes from the original excitation by light, relations based wholly on the simple laws of photochemistry may not appear to hold. For such reasons much controversy has occurred concerning Blaauw's theory. Recent work of a controversial nature has been reviewed by Brauner (1927).

From the experimental standpoint a simple situation exists in the sporangiophore of *Phycomyces*, in that the whole region of growth and of sensitivity to light is limited to a meristematic zone extending less than 2 mm. below the sporangium (Fig. 1). The whole sporangiophore is also without cross-walls, so that there is no complication due to

transportation of substances over long distances or in specialized vessels. It might be expected, therefore, that in *Phycomyces* the



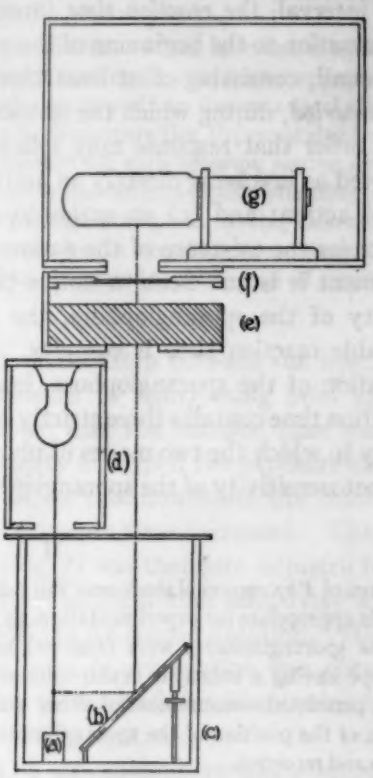


FIG. 1 FIG. 2

FIG. 1. Outline drawing of the distal extremity of a sporangiophore showing the terminal sporangium and the probable extent of the zone of growth and of photic sensitivity. As grown for these experiments, the length of the mature sporangiophore commonly becomes 80 to 100 mm., so that the sensitive, elongating region having at most an extent of 1 to 2 mm. is markedly limited.

FIG. 2. Diagram of apparatus used in adaptation and stimulation of a sporangiophore. *a*, culture of the fungus with upright sporangiophore; *b*, 45° mirror on adjustable stand; *c*, the cell with glass top and faces, immersed in the water bath; *d*, 100-watt adapting light; *e*, shutter; *f*, heat screen; *g*, 1000-watt stimulating light. The dotted lines represent the paths of light from the respective sources.

events occurring between the reception of light and the subsequent growth response should be relatively simple.

Sudden symmetrical illumination of a sensitive sporangiophore with light of sufficient intensity leads to an acceleration of growth after a definite interval, the *reaction time* (measured from the beginning of the illumination to the beginning of the response). This reaction time is compound, consisting of at least three major components: (1) an *exposure period*, during which the sporangiophore must be exposed to light in order that response may follow; (b) a *latent period* proper, interpreted as involving directly an activity of the products of photochemical action; and (c) an *action-time* necessary to the response. Evidence for the existence of the action time will be given shortly; at the moment it is sufficient to notice that in spite of the structural simplicity of the sporangiophore, the composition of the simplest measurable reaction time is complex. In the case of asymmetrical illumination of the sporangiophore, leading to phototropic response, the reaction time contains three strictly comparable major components. The way in which the two modes of photic response are related to the basic photosensitivity of the sporangiophore is therefore of interest.

II

Cultures of *Phycomyces blakesleanus* Burgeff ("+" strain) were grown in short glass vials appropriate for experimentation, as previously described (Castle, 1927–28). The sporangiophores were observed laterally by means of a horizontal microscope having a rotatable ocular micrometer scale which, depending on its position, permitted measurement of either vertical growth or horizontal bending. Readings of the position of the sporangium on the scale were made at 15-second intervals and recorded.

The glass observation cell in which the cultures were placed for experimentation had plane walls and cover (Fig. 2, *c*), and was sunk nearly to the brim in a water thermostat enclosed in a small dark-room into which the observation microscope projected. The temperature of the bath was held at 24.5 ± 0.1°C. throughout all the experiments. A culture of young, rapidly growing sporangiophores with fully formed sporangia was placed at the bottom of the moist glass chamber, and was allowed to become oriented by and thoroughly adapted to illumination of 86 ft.-candles from a 100-watt bulb directly above the culture (Fig. 2, *d*). Beside the culture in the chamber stood a 45° mirror, *b*. Light coming from a 1000-watt lamp, *g*, in a special housing above, passed through a colloidal-gold heat-screen, *f* and shutter, *e*, and was finally reflected horizontally onto the sporangiophore by the 45° mirror. This was the stimulating light, giving an illumination of 171 ft.-candles at the sporangiophore.

The adapting and stimulating lights were controlled from outside the dark-room by means of switches, and the shutter by means of a cable release, all of the controls being within reach of the observer sitting at the ocular of the horizontal microscope. In the microscope, the silhouette of the sporangium against a weak red observation light was seen superimposed on the micrometer scale.

Two types of photographic shutter were used: an Eastman focal plane shutter for relatively brief exposures, and a large aperture Ilex Universal shutter for longer exposures. Each shutter was calibrated for each exposure setting by causing a spot of light to pass through the shutter and fall on a rapidly vibrating tuning fork (250 vibrations per second). A photographic plate moving beneath the fork during the operation of the shutter received a record of the number of vibrations of the fork. On developing the plate, the record could be read off directly in units of time.

It was desired to study the relationship between the reaction time and the duration of the exposure to light, using brief exposures. Previous experiments had shown that the reaction time was practically constant for a given intensity of light if the exposure exceeded a certain minimum duration. Below that minimum, the reaction time increased progressively as the exposure time decreased. The apparatus which has been described (Fig. 2) was therefore adjusted to permit stimulation of sporangiophores of uniform high sensitivity with brief flashes of light ranging from 0.005 to 0.6 second duration. High sensitivity of the sporangiophores prior to stimulation was obtained by allowing each culture to remain in the dark for 30 minutes following complete adaptation to the orienting light.

In the actual procedure: (1) a sporangiophore was adapted to the orienting light of 86 ft.-candles for 30 minutes; (2) the orienting light was then put out, and dark adaptation of 30 minutes duration allowed to take place; (3) stimulation with unilateral illumination of 171 ft.-candles was effected for varied brief times of exposure; (4) the reaction time of the resulting growth response was determined from plottings of the position of the sporangium on the micrometer scale at 15-second intervals (see Fig. 3, *a* and *b*; also Castle, 1928–29). The first perceptible deviation from the preëxisting rate or direction of growth is taken as indicating the moment of response.

It should be noted that the stimulating illumination in all cases strikes the sporangiophore from the side, due to the 45° mirror. Even under these circumstances a direct growth acceleration typi-

cally follows, although there also occurs a practically simultaneous phototropic bending. The two modes of photic response might well

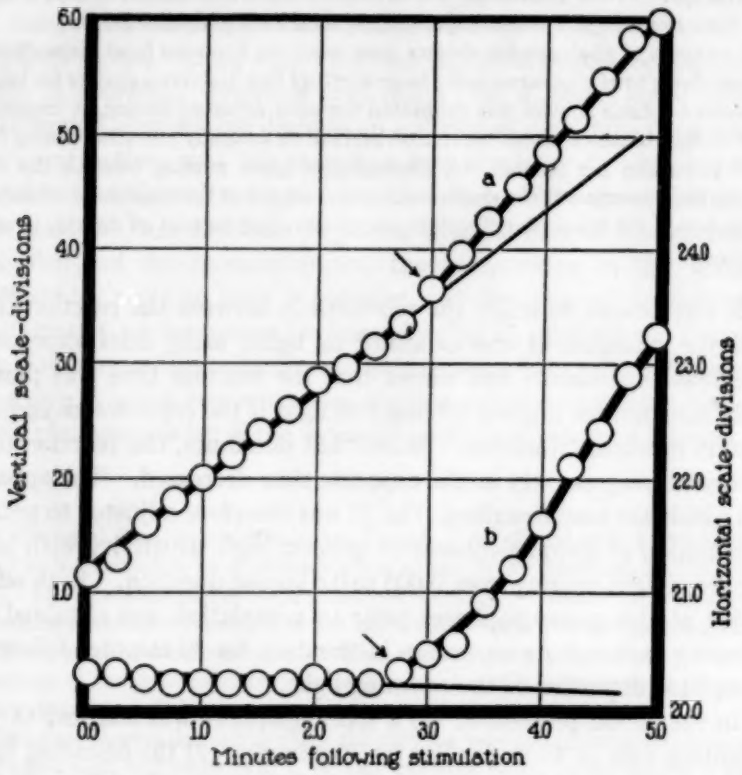


FIG. 3. Plots showing individual growth responses of each type: *a*, the *direct growth response; b*, the *phototropic growth response.* In each case, the abscissa represents the time elapsed since the beginning of stimulation, which commenced at zero on this scale. The points are individual ocular micrometer readings. The ordinates on the left represent the length of an elongating sporangiophore, *a*; those on the right the position of a sporangium, *b*, on the horizontal ocular scale. The first significant deviation from the preëxisting rate or direction of growth is taken as the moment of response, indicated in each case by an appended arrow.

be expected to go hand in hand, since to produce the usual "positive" phototropic bending (toward the source of lateral light) there must

be presumed to be greater effective action of the light on the more remote half of the sporangiophore. Yet all of this light has necessarily passed through the nearer half. Oehlkers (1926) has suggested that internal reflection within the highly refractive sporangiophore may lead to greater absorption of light in the more remote half. In any event, the conditions under which acceleration of growth and bending are compared in these experiments are identical. The *beginning* of

TABLE I

Mean reaction times of the direct growth-response and of the phototropic response to various durations of exposure to unilateral light of 171 ft.-candles. Each mean *R.T.* represents the average of from 13 to 22 determinations on individual sporangiophores. Such averaging of the photic responses is justifiable in spite of the differences found to exist between individuals in absolute rate of growth (*cf.* Castle, 1927–28; 1928–29).

Exposure (*sec.*)	Direct growth-response			Phototropic response		
	Mean *R.T.* (*min.*)	*P.E.* of mean *R.T.*	$\frac{1}{R.T.-2.50}$	Mean *R.T.* (*min.*)	*P.E.* of mean *R.T.*	$\frac{1}{R.T.-2.25}$
0.005	3.84	±0.035	0.746	3.63	±0.028	0.725
0.01	3.53	.029	0.971	3.50	.061	0.800
0.03	3.33	.042	1.21	3.38	.040	0.885
0.09	3.42	.031	1.09	2.98	.024	1.37
0.13	3.27	.041	1.30	—	—	—
0.15	2.98	.046	2.08	2.96	.030	1.41
0.20	3.13	.036	1.59	—	—	—
0.27	3.06	.057	1.79	2.76	.037	1.96
0.34	2.84	.030	2.94	—	—	—
0.38	—	—	—	2.60	.048	2.86
0.54	2.86	.045	2.79	2.61	.028	2.79
0.6	2.74	.02	4.17	—	—	—

either response is the measured end-point, and the position of the ocular scale, whether vertical or horizontal, determines which response shall be observed.

III

Sensitive sporangiophores stimulated at constant temperature by unilateral light of constant intensity but of varied brief duration exhibit a regular relationship between the reaction time and the dura-

tion of the exposure to light. This holds for both growth acceleration and phototropic bending. The two sets of data are given in Table I, plotted separately in Figs. 4 and 5. The reaction time figures are averages of from 13 to 22 individual determinations. The justifica-

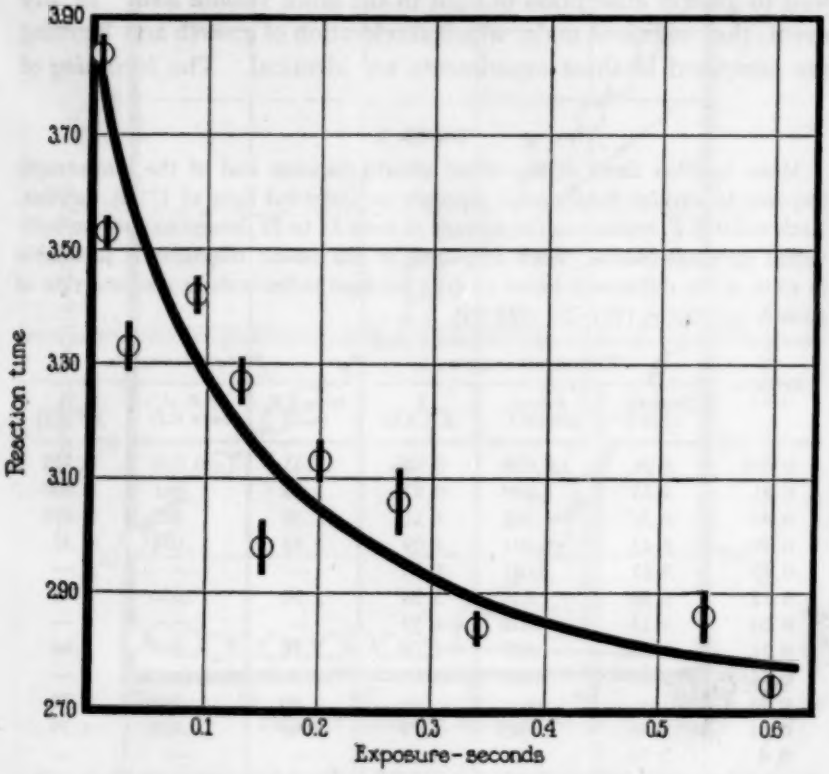


FIG. 4. Mean reaction times of sporangiophores of *Phycomyces* as a function of duration of exposure to light, measured by means of the direct growth response. Each point is the average of from 13 to 22 individual determinations, twice the probable error of the mean being given by the height of the vertical bar through the circles. The curve is arbitrary, but identical with that in Fig. 5.

tion of such averaging of photic responses in spite of the differences found to exist between different sporangiophores in absolute rate and in the temperature relations of growth (Castle, 1927–28) lies in the

small size of the probable errors. The comparability as concerns photic responses of different sporangiophores grown under similar conditions is striking evidence of the relative autonomy within the sporangiophore of the photosensitive system.

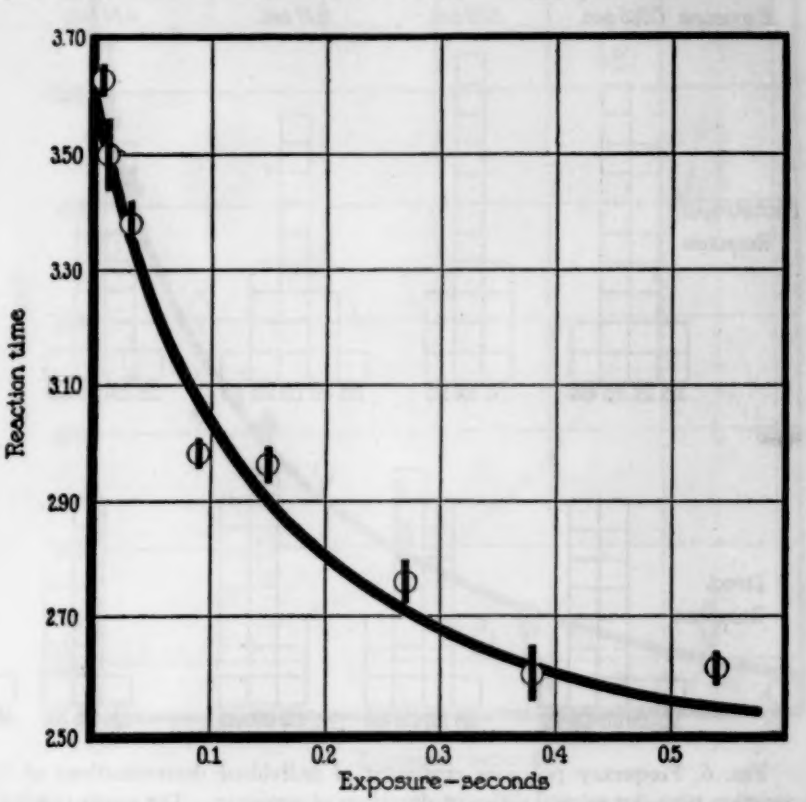


FIG. 5. Mean reaction times of sporangiophores of *Phycomyces* as a function of duration of exposure to light, measured by means of the phototropic growth response. Each point is an average, the height of the vertical bar indicating twice the probable error of the mean. The curve is arbitrary, but identical with that in Fig. 4.

The actual frequency distributions of the individual growth responses for several different exposure times are given in Fig. 6. The magnitude of the "class range" may be thought to be relatively large

(one-quarter minute), but it is determined wholly by the method of experimentation, which does not permit readings to be made more frequently than four to a minute.

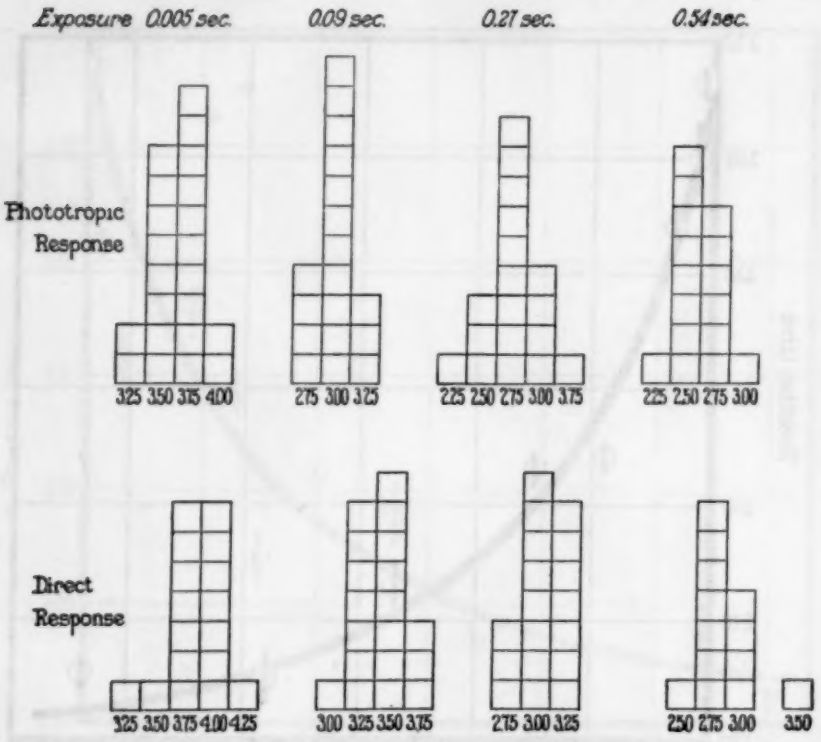


FIG. 6. Frequency polygons composed of individual determinations of the reaction time, for several different durations of exposure. The upper row is for the phototropic response, the lower for the direct growth response. The displacement of the upper series to the left by approximately one unit (0.25 minutes) relative to the lower is evident.

The same curve may be made to fit the points from both the sets of data, provided a constant time (15 seconds) is added to the reaction times for the phototropic response (Fig. 7). This constant is the time interval at which successive readings of the position of the sporangium

on the ocular scale are made. The fact that the reaction time figures from the phototropic response are, on the whole, 15 seconds less than those from the other series is merely evidence that as judged from the

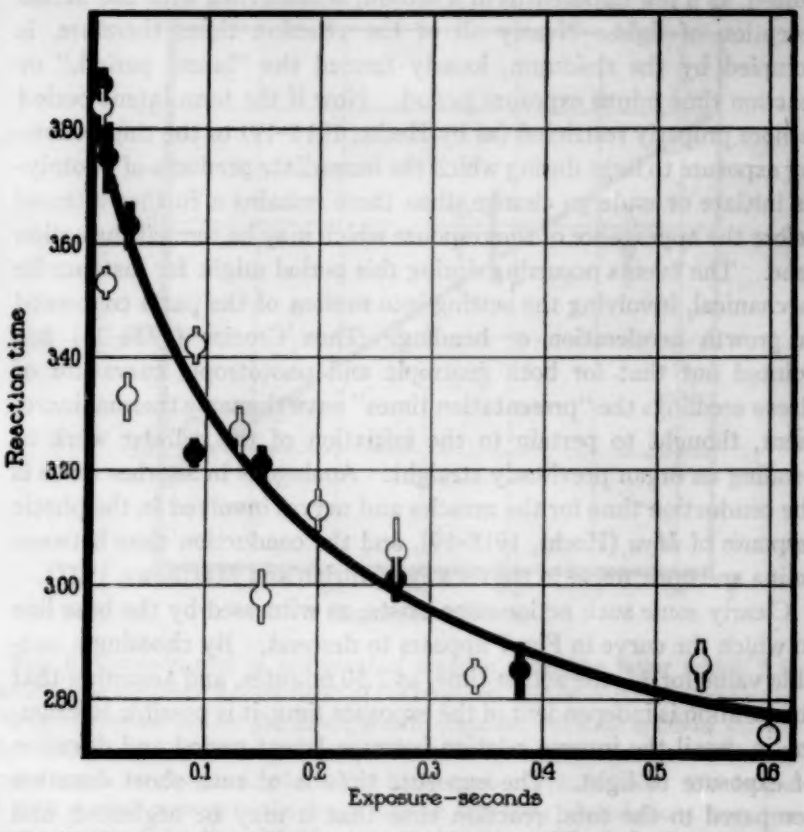


FIG. 7. Common plot of the mean reaction time data for both the direct response (open symbols) and the phototropic response (solid symbols). To the latter 0.25 minutes has been added in making this plot, for the reason explained in the text. The curve is a hyperbola, one constant of which is taken from the slope of the line in Fig. 8. The height of each symbol represents twice the probable error of the mean.

plots the phototropic response begins more sharply, and the first perceptible response is estimated one unit (15 seconds) earlier than the more gradually appearing growth acceleration.

It is desired to gain some insight into the possible nature of the secondary processes taking place during the total reaction time. Of this long period, often lasting 3 minutes or more, a very short interval indeed, as a few hundredths of a second, is concerned with the actual reception of light. Nearly all of the reaction time, therefore, is occupied by the residuum, loosely termed the "latent period," or reaction time minus exposure period. Now if the term latent period is more properly restricted (as by Hecht, 1918–19) to the time following exposure to light during which the immediate products of photolysis initiate or undergo change, then there remains a further interval before the appearance of the response which may be termed the action time. The events occurring during this period might for instance be mechanical, involving the setting into motion of the parts concerned in growth acceleration or bending. Thus Crozier (1924–25) has pointed out that for both geotropic and phototropic curvature of *Avena* seedlings the "presentation times" have the same thermal increment, thought to pertain to the initiation of the cellular work of bending an organ previously straight. Analogous in another sense is the conduction time for the muscles and nerves involved in the photic response of *Mya* (Hecht, 1918–19), and the conduction time between retina and optic nerve in the eel's eye (Adrian and Matthews, 1927).

Clearly some such action-time exists, as witnessed by the base line to which the curve in Fig. 7 appears to descend. By choosing a suitable value for M, the action-time, as 2.50 minutes, and assuming that its duration is independent of the exposure time, it is possible to examine in detail the inverse relation between latent period and duration of exposure to light. The exposure time is of such short duration compared to the total reaction time that it may be neglected, and reaction time minus action-time ($R.T.-M$) may be used instead of latent period minus action-time ($L.P.—M$). Therefore, the velocity of the process occurring during the latent period may be expressed by the reciprocal, $1/(R.T.—M)$. Taking M as 2.50 minutes, and plotting $1/(R.T.—M)$ against t, the exposure time, a linear relationship is found to exist between the two variables (Fig. 8), holding equally for both of the modes of photic response and further indicating their basic similarity. It is interesting that this linear relationship is similar

to that which has been found for the duration of the latent period in the response of the clams *Mya* and *Pholas* to light (Hecht, 1918–19; 1927–28) and for the latent period in the electrical response of the

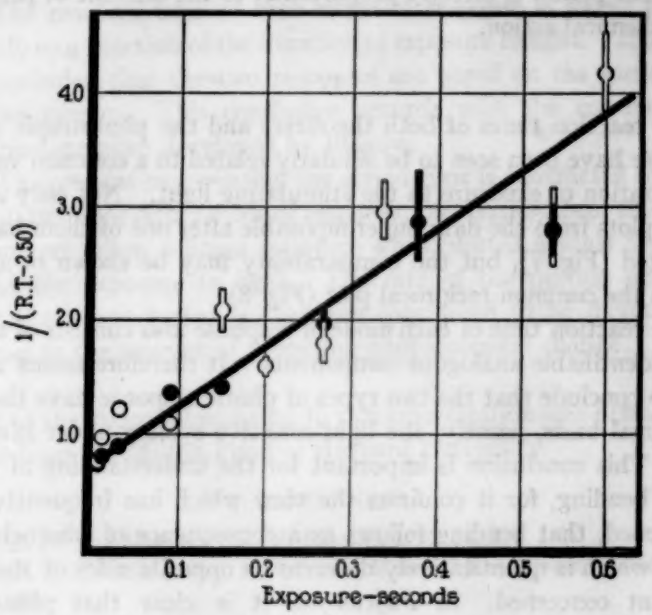


FIG. 8. Common plot of reciprocal of reaction time (the latter minus 2.50 minutes, the apparent "action-time" of the response) against duration of exposure. The open symbols are for the direct growth response, the solid symbols for the phototropic response. The phototropic response is corrected for its consistently earlier appearance by plotting $1/(R.T.-2.25)$ instead of $1/(R.T.-2.50)$. The height of each symbol represents the magnitude on this scale of deviations from the mean equal to twice the probable error of the mean. When these are smaller than the size of the circles, they are not indicated. In this reciprocal plot the absolute size of these deviations of course increases rapidly as the ordinate increases; the goodness of fit of the line is not invalidated by this.

eel's retina to light (Adrian and Matthews, 1927). The relation may be interpreted in a number of ways. The assumptions made in the case of *Phycomyces* are that M has a constant value of 2.50 minutes, and that for short exposures the amount of photochemical action

varies directly with the duration of the exposure. Within these assumptions the relation certainly indicates that over the range of exposure times studied the velocity of the process occurring during the latent period is directly proportional to the amount of preceding photochemical action.

IV

The reaction times of both the direct and the phototropic growth response have been seen to be similarly related to a common variable, the duration of exposure to the stimulating light. Not only are the direct plots from the data superimposable after one of them has been corrected (Fig. 7), but the comparability may be shown in another way in the common reciprocal plot (Fig. 8).

The reaction time of each mode of response also consists of at least three identifiable analogous components. It therefore seems reasonable to conclude that the two types of photic response have the same functional basis, namely, the light-sensitive system under investigation. This conclusion is important for the understanding of phototropic bending, for it confirms the view which has frequently been questioned, that bending follows as a consequence of photochemical action which is quantitatively different on opposite sides of the organ or plant concerned. In *Phycomyces* it is clear that phototropic bending results from local differences in the action of light, and that the action occurs in the same light-sensitive system which also produces the ordinary, direct growth response.

SUMMARY

1. The reaction time of the direct growth response of the sporangiophore of *Phycomyces* to light consists of a series of at least three major identifiable components: (*a*) an *exposure period* during which photochemical change occurs; (*b*) a *latent period* involving products directly consequent upon the photochemical action; and (*c*) an *action-time* occupying a further interval before the growth acceleration appears.

2. The reaction time of the phototropic response of the sporangiophore following stimulation by unilateral illumination is also compound, and is made up of at least three components comparable to those of the direct growth response.

3. The reaction time of each mode of response is constant for a particular intensity of illumination, provided that the duration of the exposure period exceeds a certain value. Below that value the reaction time increases progressively as the exposure time decreases.

4. The reaction time of each mode of response is found to vary similarly as a function of the duration of exposure to light. It is therefore concluded that the two responses are based on the same light-sensitive system. This conclusion accords with the conception of plant phototropism developed by Blaauw.

5. If a constant representing the action-time is subtracted from the reaction times for either mode of response, the reciprocals of the resulting numbers follow a linear sequence when plotted against the durations of the exposure to light. The rate of the process occurring during the latent period is therefore considered to be directly proportional to the amount of preceding photochemical change.

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THE PERMEABILITY OF THE SURFACE OF MARINE ANIMALS

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The opinion that the surfaces of aquatic animals are semi-permeable was chiefly based on the researches of Frédéricq and of Bottazzi, which were carried out on marine forms, and on the experiments of Overton on amphibians. According to these experimenters water alone penetrates the skin, while dissolved substances will not pass through it unless they are soluble in lipoids. Only in a few cases, however, was proof furnished by chemical means that substances dissolved in water do not penetrate into the body of the experimental animals. In the main, in the past reliance was placed on the results of experiments which measured osmotic changes alone. I may be permitted to discuss briefly such experiments.

In accordance with the statements of Frédéricq and of Bottazzi (1) the osmotic pressure of most marine animals save fishes is equal to that of the surrounding sea water. If such marine animals are put into more diluted or more concentrated sea water, the content of salt in the blood and hence its osmotic pressure decreases or increases correspondingly in a very short time.

At the same time as the osmotic pressure changes in diluted sea water, according to Frédéricq and others, the volume of the animals increases and in more concentrated sea water decreases. Consequently the deduction readily was drawn that the equalization of the internal with the external osmotic pressure was produced by the diffusion of water alone. A proof of this presumption could only be given, however, if the increase in volume takes place in all animals and corresponds to the difference in osmotic pressure. But this is not the case, as I will subsequently show.

To examine the permeability of the surface of animals for substances

dissolved in water, it is best to choose substances which exist both within the animal and in its environment. These are the ions of sea water. But one has to avoid the danger of complicating the process of equalization by causing changes in the osmotic pressure. This is only possible by using artificial sea water, in which one ion alone is either increased or diminished.

I chose for my experiments the same species of animals employed by Frédéricq and by Bottazzi, the common crab (*Carcinus maenas*) representing the crustaceans, the sea-slug *Aplysia* representing the molluscs.

When these animals were kept in artificial sea water containing no Ca, the Ca-content of their blood decreased constantly, as I have shown in a previous paper (2). In the crab it diminished to one-third in about 10 days, in *Aplysia* in 5 hours. If I used artificial sea water free from Mg or K, the content of these ions in the blood decreased in the same way. On the other hand the Ca (Mg or K) in the blood can be augmented, when the animals are kept in sea water with a high content of these ions.

All these facts *show that the surfaces of marine invertebrates are not impermeable for ions* (or their salts) as suggested by Frédéricq, Bottazzi, and Macallum.

Macallum (3) based his hypothesis on his discovery that the body fluids of marine animals living in sea water of varying concentration, always show the same relative relationship of the ions one to another, which differs in a specific manner from the relationship of the same ions in the sea water. I convinced myself of the correctness of this statement in regard to crabs, *Aplysia*, and other marine forms by transferring them from ordinary sea water into sea water either diluted or concentrated. As the relative concentrations of the ions in the outside medium did not change in such experiments, it does not change within the body either. Only when the relation in sea water is changed will it change in the blood also.

On the other hand there were some experiments on the changes in osmotic pressure from which Frédéricq and Bottazzi drew the conclusion that the surface of all marine invertebrates has the quality of a semi-permeable membrane. I was obliged to try to bring their results into harmony with my own, which showed that the surfaces are permeable also for dissolved substances as, for example, for salts.

If, when one brings an animal from ordinary sea water into a more diluted or a more concentrated one, the compensation for the change in osmotic pressure took place merely by the transport of water inwards or outwards, the volume of the animal would have to change. The amount of such change could be calculated beforehand, and the new volume assumed would have to remain constant as long as the animal remained in the altered sea water. Neither the one nor the other condition is fulfilled.

Let us see what experiments with *Aplysia* will show. If we place it in half-diluted sea water the weight should double. But at best it increases only about 60 per cent (Fig. 1). Very soon the weight decreases again and the original weight is nearly reached after 6 to 10 hours. When less diluted sea water (relation of 3 to 1) is used the situation is still clearer. After increasing in weight at first, it decreases again, in many cases after 2 hours, and returns to the original weight in another 2 or 3 hours. The weight may even decrease still further. On transferring the animal into normal sea water, a new and rapid diminution of weight occurs. But a short time later the weight becomes constant (Fig. 1).

These changes of weight are only explainable if in addition to a transfer of water a transfer of salt from within to without also occurs. To ascertain this more precisely, samples of blood were taken at various times after transfer into diluted sea water. As we had found, as stated above, that the relative relations of the blood constituents did not change, it was not necessary to make a complete analysis in each case but an estimate of the Cl content would be sufficient; and this was calculated from the blood sample taken as if the animal consisted of blood alone. On the basis of these experiments it was shown that the increase in weight at the beginning was almost entirely due to the transfer of water alone. But later on considerable amounts of salt, even up to 37 per cent, are lost. In this manner the animal comes into equilibrium with the external osmotic pressure. The lost salt can be recovered almost completely from the outside water. When put back into normal sea water, first a loss of weight owing to transfer of water occurs, which is followed by a pretty rapid transfer of salt to the inside of the animal. This taking up of salt can also be easily shown by blood analyses.

Aplysia, on being transferred from normal sea water to a more concentrated one, very quickly decreases in weight, but this loss of weight remains behind that arrived at by calculation. After a short time the weight increases again and becomes constant. But in none of the experiments hitherto performed has the original weight been

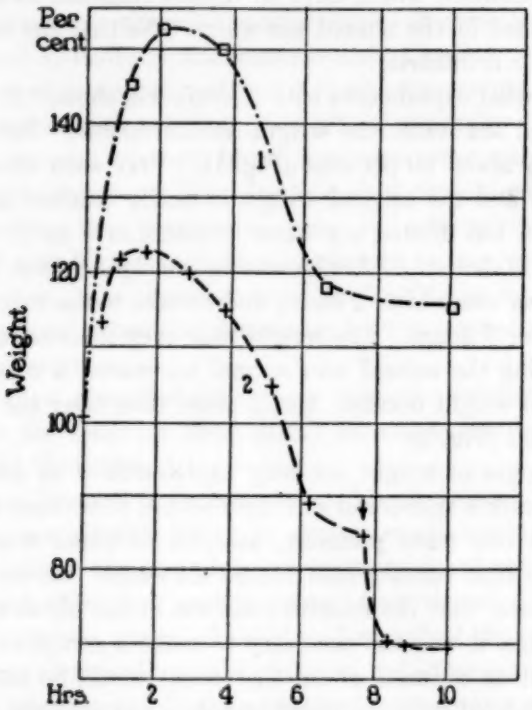

FIG. 1. Change of weight of *Aplysia* in diluted sea water: (1) half concentrated, (2) three-fourths concentrated. ↓ normal sea water.

regained. In this case, too, calculation shows that not only does water leave the body but salt also enters it.

In fact, the *compensation to osmotic-pressure changes* in *Aplysia* takes place *in large part by transport of water*. But this is not the only cause. In *addition a transport of salt takes place*, which is only revealed by exact analysis.

In the hard-shelled crabs the reactions are quite different. Here a

transfer of water is difficult to prove. Within the limits of the method there is *no change of weight.* Hence the *compensation for the osmotic-pressure changes* in hypotonic or hypertonic solutions *can only take place by transfer of salt.* It hardly could be expected that the result would be otherwise. If the compensation were to be effected by water transfer, the animals in diluted sea water would be likely to burst as there is no room within the shell for the entering water. Or in concentrated solutions a negative pressure would be established within the carapace, which can not shrink.

The experiments performed up to the present make it evident that osmotic pressure can not be the only effective force producing the compensation which occurs on transferring the animals into more or less concentrated sea water. But in addition it seems that the compensation is in part produced by the salts or their ions, in obedience to the laws of diffusion, passing through the skin from places of higher to that of lower concentration. This question can be decided by very simple experiments.

Animals transferred to sea water, diluted not with distilled water but *with a solution of a non-electrolyte isotonic with sea water, ought not to change their weight* as the osmotic pressure of the outside solution should remain equal to that of the blood. But in fact all species of *soft-skinned animals* experimented with *shrunk in such solutions permanently,* till at last their blood became so viscous that death occurred. On the contrary, hard-skinned animals which are unable to shrink die much faster because they lose nearly all their blood. The death of some marine animals in isotonic solutions of cane sugar, often referred to in the literature, does not depend, as has been generally accepted, upon the toxicity of pure sugar solutions but on the concentration (thickening) of the body fluids.

I am now going to report on experiments of this kind. I will speak first about the soft-skinned animals covered with a mucous membrane and again will take *Aplysia* as my example. Figure 2 shows the loss of weight of *Aplysia* on being transferred into sea water diluted either to one-quarter or one-half with an isotonic solution of cane sugar. Both curves are from the averages of a large number of experiments. You see how in the one case the weight has decreased to one-half after as little as 6 hours, and in the other case after 18 hours, and this

in spite of the fact that the osmotic pressure was the same outside and within the animal. After losses of weight of such an extent the blood

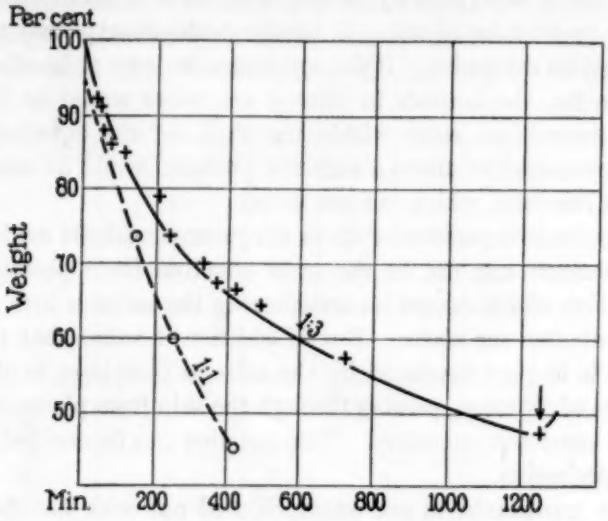

FIG. 2. Loss of weight of *Aplysia* in a mixture of isotonic cane sugar solution and sea water. ↓ normal sea water.

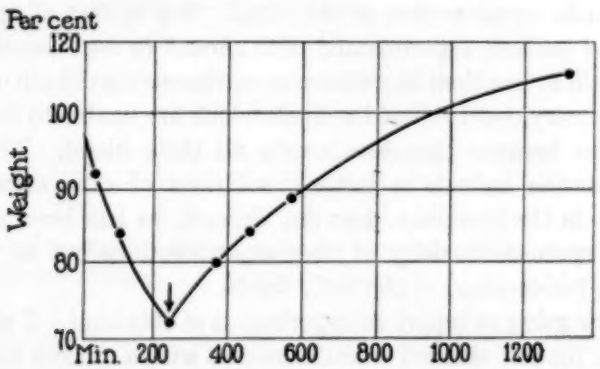

FIG. 3. Loss of weight in 1 part of cane sugar solution and 3 parts sea water, and recovery in sea water ↓.

has become so thick that it can hardly be squeezed out after cutting the skin. In normal animals it is quite liquid and flows out by itself quite easily when they are wounded. The Cl-content does not de-

crease much relatively, but calculated on the weight of the animal it is quite considerable up to 50 per cent.

These occurrences may be accounted for as follows: The osmotic pressure is equal outside and inside, but the salt concentration is lower outside than within. As the surface is permeable for salt, salt begins to diffuse out. As the result of this, the osmotic pressure becomes lower inside than outside and water passes to the outside. And this procedure is repeated again and again and an equilibrium

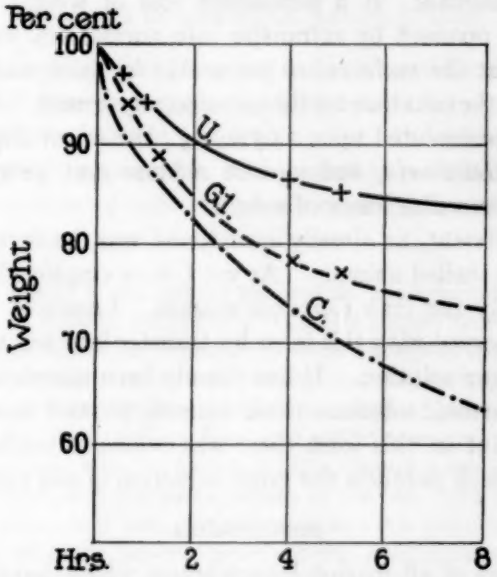

FIG. 4. Loss of weight in 1 part isomolecular solution of urea (U) or glycerine (Gl) or cane suger (C) and 3 parts of sea water.

can not be achieved. Theoretically the animals would lose all their water if the volume of the external solution were sufficiently great. The process ceases because the circulation of the blood stops.

If the animals are transferred at the right time into normal sea water, they swell quickly again, regaining from the sea water salt as well as water (Fig. 3). In some cases it may be noted that the original weight is even exceeded. This permits the assumption that the skin is not quite impermeable for cane sugar. Indeed, it can be shown by blood

analysis that this non-electrolyte does enter to a certain extent. *Other non-electrolytes penetrate more quickly*, dextrose more so than cane sugar, glycerine still more rapidly, and urea more rapidly still. In accord with this, in equal time periods, the losses of weight are smaller with urea than with glycerine and still smaller than with cane sugar (Fig. 4).

Experiments were made with this method of transfer into sea water diluted with isotonic non-electrolyte solutions with other species of soft-skinned animals. If a permanent loss of weight takes place, which can be reversed by retransfer into normal sea water, there is clear proof that the surfaces are permeable for salts, and much more permeable for the salts than for the non-electrolyte used. All the species of animals experimented upon as yet, *e.g.*, the worm *Sipunculus*, the Echinoderm *Holothuria*, and various medusa and pelagic molluscs, showed such reversible losses of weight.

Entirely different, as already mentioned, are the facts ascertained with the hard-shelled animals. As yet I have employed for these experiments only the crab *Carcinus maenas*. Losses of weight could hardly be observed with this form by transfer into sea water diluted with cane sugar solution. It has already been mentioned that even in highly hypertonic solutions these animals showed hardly any loss of weight. But in this form there was evident a marked *thickening of the blood* which parallels the great reduction in salt content.

CONCLUSIONS

The surfaces of all marine invertebrates which have been experimented upon are permeable for water and also for both the salts or their ions which are in solution in their blood and in sea water. The forces which tend to bring the salt content of the blood into equilibrium with the salt content of the surrounding sea water are just as great as the forces which strive to prevent osmotic differences. The skin of these animals, save in the cases where special modifications have arisen, serves only as a protecting barrier preventing the loss of the body colloids.

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THE CONCENTRATION EFFECT WITH VALONIA: POTENTIAL DIFFERENCES WITH CONCENTRATED AND DILUTED SEA WATER

By E. B. DAMON and W. J. V. OSTERHOUT

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The concentration effect promises to be of considerable importance as a means of revealing properties of protoplasm otherwise unsuspected. In view of this, a series of studies has been started, one of which has recently appeared.[1] The present paper deals with the P.D. between the interior of cells of *Valonia macrophysa* and concentrated or diluted sea water applied externally.[2]

In an earlier paper,[3] it was pointed out that in measurements of P.D. across the protoplasm in *Valonia*, the presence of parallel circuits through the cell wall may cause serious difficulties. These make it undesirable to apply two different solutions simultaneously at more or less widely separated spots on the same cell.[4] In the present experiments we have established connection[3] with the interior of the cell through a fine glass capillary filled with artificial sap, on which the cell is impaled, while contact with the outside is made through a continuous stream of solution which flows over the entire surface of the cell. The protoplasm thus comes into the circuit only once, and the P.D. across it is measured directly. On standing, the protoplasm becomes sealed to the glass capillary, preventing a possible short circuit between the external solution and the vacuolar sap through a

[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761.

[2] The experiments reported in this paper were carried out at Bermuda. The apparatus and experimental technique have been described in earlier communications.[3] The Compton quadrant electrometer used in these measurements, however, was an improved model made by the Cambridge Instrument Company. This was adjusted to give deflections of 3.5 to 5.0 mm. per mv. with the scale 4 feet from the mirror.

[3] Damon, E. B., *J. Gen. Physiol.*, 1929–30, **13**, 207. See also Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, **11**, 193.

[4] With *Nitella*, however, and probably also with other fresh water plants, the resistance of the cell wall is so high that such parallel circuits may be shown to be of only negligible importance.

film of solution between the protoplasm and the glass. We believe that the values measured when there is a good seal actually represent the E.M.F. acting across the protoplasm of the impaled cell (leaving open the question of how much the cell may be altered by impalement[5]).

We apply a series of different solutions to the same cell, and compare the P.D.'s to which they give rise. By combining the values observed in actual measurements on chains of the type:

sap (inside) | protoplasm | solution *A*

and

sap (inside) | protoplasm | solution *B*

we may calculate the E.M.F. of the chain:

solution *A* | protoplasm | sap | protoplasm | solution *B*

for the ideal case (*i.e.*, in which parallel circuits through the cell wall play no part).

The following convention which we have adopted for reporting the signs of the P.D.'s may assist in avoiding confusion as to which type of chain is meant. For all measurements between an external solution and the vacuolar sap, made with the use of a capillary, the sign of the interior of the cell is reported, thus: "inside positive" or "inside negative." Such expressions as "sea water positive" or "solution at *A* positive" are reserved for actual or fictitious chains in which we lead off from two different points on the outside of the cell. In either case, the solution or spot reported as positive is the one from which positive current tends to flow through the measuring instrument in the external circuit.

In case the P.D.'s fluctuate so as to indicate that the system is changing continuously during the measurement or the solution first applied produces in the cell alterations which affect its behavior in later measurements,[6] we may compare the values with a given solution, measured on one series of cells with those of another solution measured on a different series; in this case, however, variations among individual cells must be considered.

The concentration effect with sea water presents an especially favorable case for comparing measurements with a series of solutions on the

[5] *Cf.* Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

[6] Experiments with artificial sap[3] illustrate these difficulties.

same cell, for the observed changes in P.D. are regular and reproducible within certain limits. If the solution is too dilute, or its application too long continued, secondary changes occur in the cell which result in the change of the sign of the P.D. These changes are reversible up to a certain point, beyond which they become irreversible, involving permanent injury. These secondary changes are delayed by making the dilute solutions isotonic with ordinary sea water by the addition of glycerol.[7] Further studies of these secondary changes are being carried on.

Changes in the protoplasm produced by the solutions applied to the cells are always a source of difficulty. An advantage of the capillary method is that it makes these changes less liable to be overlooked or misinterpreted.[8]

The sign of the concentration effect with sea water, *i.e.*, the sign of the E.M.F. of the (imaginary) chain:

sea water, concn. 1 | *Valonia* | sea water, concn. 2

is made evident by the changes in P.D. across the protoplasm observed when sea water is replaced by concentrated or diluted sea water. We find that, until secondary changes begin to appear, the more

[7] In practice, the sea water is diluted with a solution of glycerol, 8.7 per cent by weight, in distilled water. The freezing point of this solution is approximately the same as that of Bermuda sea water. We have used sugar solutions, also, but prefer glycerol since it is less liable to be contaminated by molds.

[8] For example, we have tried measurements on intact (unimpaled) *Valonia* cells, in which we led off from one end with sea water, and from the opposite end with sea water diluted with 8.7 per cent glycerol solution, using flowing contacts. When recently collected cells were employed, we found that the more concentrated solution was positive, but (probably as a result of short circuits through the cell wall) the P.D.'s were smaller than in measurements by the capillary method. The observed P.D.'s could be somewhat increased by wetting the greater part of the surface of the cell with dilute sea water, instead of ordinary sea water. In either case, secondary changes, reversing the sign of the P.D., occurred much sooner than in measurements by the capillary method. When we used apparently healthy cells which had been standing for some time in sea water in the laboratory, the observed P.D.'s were still smaller, and secondary changes set in more promptly. As a result, in many cases, only the P.D. after the reversal of sign was observed. Such experiments alone would have led to reporting the wrong sign for the concentration effect.

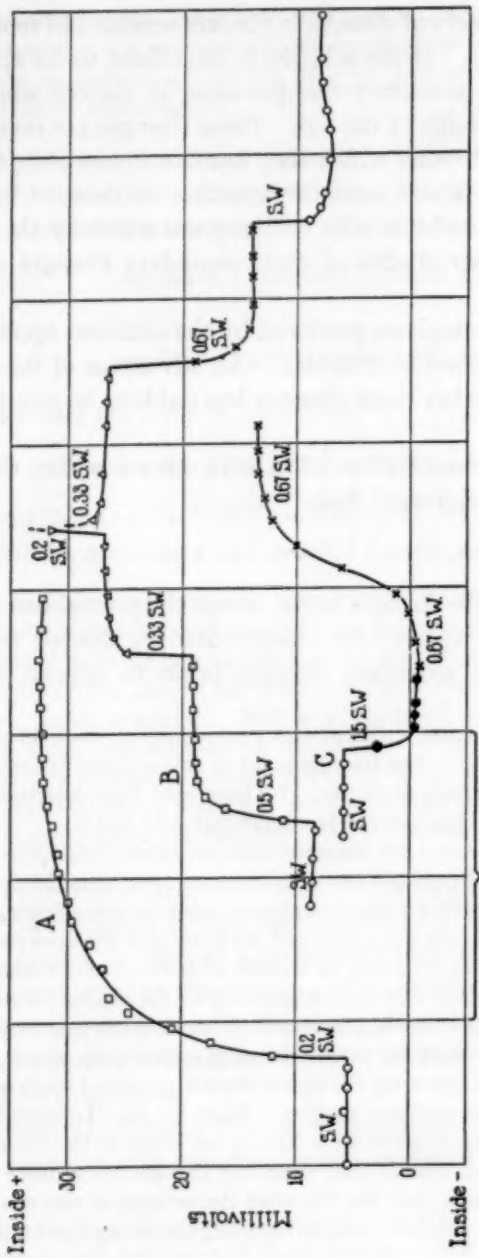

FIG. 1. P.D.-time curves, showing the concentration effect in *Valonia* with concentrated and diluted sea water. The ordinates represent the P.D. between the interior of the cell and the external solution indicated. Curve *B* represents measurements on cell No. 2, Curves *A* and *C* on cell No. 3 of Table I.

concentrated solution is positive (*i.e.*, positive current tends to flow in the external circuit from the more concentrated to the more dilute solution).

The relation between concentration and P.D. is best studied in the range between 1.5 sea water to 0.2 sea water. These concentrations probably represent the limits within which values can be obtained

TABLE I

Effect of Concentration of Sea Water on P.D. *in Valonia**

	Concentration of sea water†					
	1.5	1.0	0.67	0.5	0.33	0.2
	mv.	mv.	mv.	mv.	mv.	mv.
1	−6.6	(0.0)‡	5.3	11.8	18.9	25.2
2		(0.0)	6.0	10.6	18.5	23.8
3	−6.2	(0.0)	7.7	11.3	19.6	27.0
4	−6.0	(0.0)	6.7	11.6	18.1	25.8
5	−5.8§	(0.0)	6.3	11.8	19.5	24.3
6		(0.0)	6.6	11.0		
Average.....	−6.1	(0.0)	6.4	11.4	18.9	25.2

* These measurements were made at Bermuda between Nov. 29 and Dec. 13, 1928. The temperature of the laboratory at this time varied between 17° and 22°C., avg. 20°C. The cells used in these measurements were collected Nov. 19, 1928.

† Expressed in terms of ordinary sea water. *I.e.*, 0.2 means a fivefold dilution; 1.5 means sea water concentrated by evaporation to two-thirds its original volume. All dilutions were made with 8.7 per cent glycerol solution.

‡ The observed P.D.'s with sea water varied between 5.1 and 8.5 mv., average 6.2 mv., inside of the cell positive.

§ Secondary changes began before the P.D. became constant. 5.8 mv., representing the maximum observed change, is probably not the full value.

without too great errors from secondary changes beginning before the full value of the P.D. has been reached. Even within these limits it is not advisable to expose a cell continuously to dilute or concentrated sea water for very long periods, but to return it to sea water between short series of measurements. In order to postpone secondary changes until after the full value has been attained, the dilute solutions must be made isotonic with sea water by the addition of glycerol (or some other

suitable non-electrolyte). Under these conditions, the P.D.'s come to definite values at which they remain reasonably constant (see the P.D.-

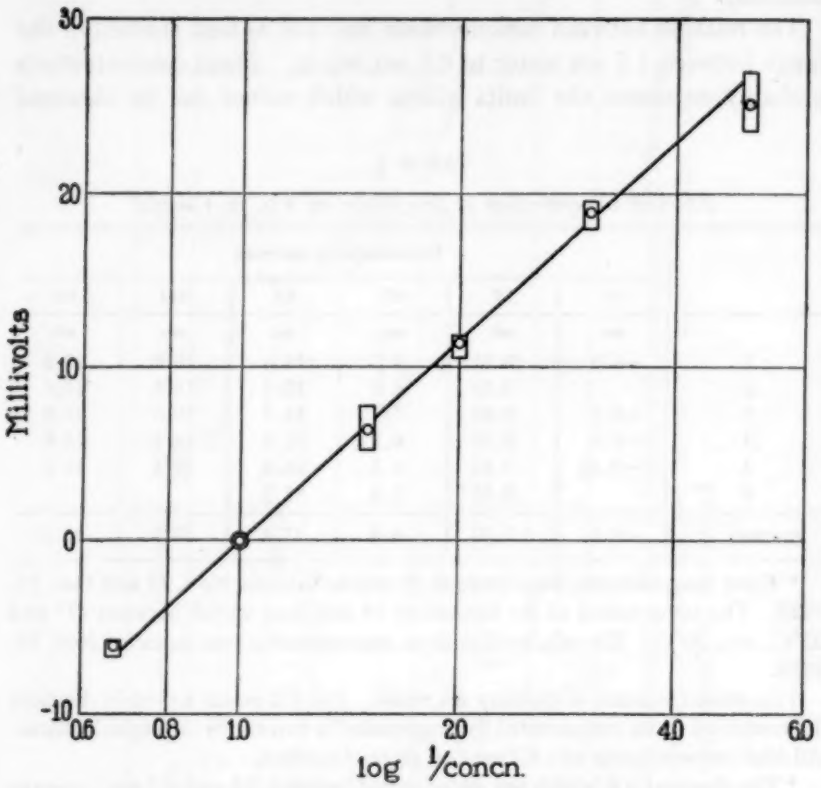

FIG. 2. Graph showing the relation between the concentration of the sea water applied externally and the P.D. across the protoplasm in *Valonia*. (See Table I.) P.D.'s plotted as ordinates are differences between the value measured with the concentration in question and the value observed with sea water. The height of the rectangles shows the variation in the observed values: averages are shown by circles. The slope of the straight line is $\frac{2}{3}\frac{RT}{F}$, where $T = 273 + 20°$, the average temperature.

time curves, Fig. 1). The constant values observed with intermediate concentrations may be approached from either side, *i.e.*, it makes no difference whether the solution in question follows a more concentrated

or a more dilute solution. The values observed with different cells are in reasonably good agreement.

These points are illustrated by the P.D.-time curves in Fig. 1, which represent measurements on two different cells,[9] and by Table I, in which are collected the P.D.'s observed with these cells and with four others. The values reported in the table are differences between the P.D. observed with the solution in question and the constant value previously or subsequently observed with sea water. In other words, the table gives the E.M.F. calculated for the chain:

sea water | *Valonia* | concentrated or diluted sea water

The sign reported is that of ordinary sea water: it will be noted that the more concentrated solution is in every case positive.

The data recorded in Table I are also shown in the graph, Fig. 2, in which the P.D. is plotted against the logarithm of the reciprocal of the concentration. The variation in the observed values is indicated by the height of the small rectangles; averages are shown by circles. Evidently the P.D. is a logarithmic function of the concentration. The straight line drawn through the observed points corresponds to the empirical equation:

$$E = \frac{2}{3}\frac{RT}{F}\ln\frac{\text{concn.}_1}{\text{concn.}_2} \tag{1}$$

for the average temperature, 273 + 20°. In other words, the P.D. across the protoplasm when the outside of the cell is in contact with concentrated or diluted sea water is given by the expression:

$$\text{P.D.} = \text{P.D.}_{\text{s.w.}} + \frac{2}{3}\frac{RT}{F}\ln\frac{1}{\text{concn.}} \tag{2}$$

where $\text{P.D.}_{\text{s.w.}}$ is the observed P.D. across the protoplasm for the cell in question when the external solution is sea water.

[9] It will be noted that when the external solution is changed the rise (or fall) of the P.D. to its new value is rather slow. This delay represents the time necessary for salts to be leached out of the cell wall (or to diffuse in) so that the concentration there is the same as in the external solution. The maximum effect cannot be observed until the salts in the entire cell wall have been brought to the new concentration.

This result (dilute solution negative) is unlike that hitherto found[10] for living cells,[11] although some non-living structures[12] show a concentration effect with dilute solution negative. This might suggest a difference between *Valonia* and the other organisms hitherto investigated, perhaps analogous to that found by Beutner[13] between acid "oils" (which give dilute solution positive) and basic "oils" (which give dilute solution negative). It is not certain, however, that *Valonia* behaves like Beutner's basic "oils" in giving with all salts a concentration effect in the same direction. We have some evidence that with KCl the dilute solution may be positive. Unfortunately, in studying the concentration effect in *Valonia* with solutions other than sea water, secondary changes are encountered which make the data hard to interpret.

Beutner found that with "oils" in most cases the concentration effect is less at higher concentrations than at low concentrations, a number of "oils" showing with the more dilute solutions the theoretical maximum of 58 mv. for a tenfold dilution. So also, *Nitella*[1] gives about 56 mv. for KCl between 0.01 and 0.001 M, but much less between 0.1 and 0.01 M. This might suggest that with *Valonia* higher values might be expected if we could employ lower concentrations of sea water. There is no hint of this, however, in the P.D.'s which we observe with concentrations down to 0.2 sea water, which proves to be the greatest dilution which we can apply to *Valonia* without inducing too rapid secondary changes.

[10] For the literature, see Osterhout and Harris.[1] With *Nitella*, dilute sea water is positive to more concentrated sea water (unpublished experiments).

[11] The only exception is the result reported by Brooks and Gelfan for *Nitella* (Brooks, S. C., and Gelfan, S., *Protoplasma*, 1928, **5**, 86) on applying sap, which is the opposite of that found for K salts in *Nitella* by Jost (Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch., Abt. B.*, 1927, Abhandl. 13, Nov.) and for sap and for NaCl and for many salts (unpublished experiments), and especially for KCl, by Osterhout and Harris;[1] in these experiments the dilute solution was positive in all cases.

[12] *E.g.*, dead frog skin at low pH values (Amberson, W. R., and Klein, H., *J. Gen. Physiol.*, 1927–28, **11**, 823), the chorion of the egg of the marine fish *Fundulus*, at low pH values (Sumwalt, M., *Biol. Bull.*, 1929, **66**, 193), collodion mixed with rhodamine (Mond, R., and Hoffmann, F., *Arch. ges. Physiol.*, 1928, **220**, 194).

[13] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben. Stuttgart, 1920.

According to Höber the protoplasmic surface of *Valonia* has some areas which are permeable to cations and other areas permeable to anions.[14] The fact that the dilute solution is negative would be interpreted to mean that the areas permeable to anions dominate the situation, and by analogy with similar rhodamine-collodion membranes the concentration effect should fall off rapidly with increasing dilution. It is evident from our results that this is not the case. There are other objections to Höber's theory which need not be mentioned here.[15]

The empirical equation (1) expressing the concentration effect with sea water in *Valonia* obviously resembles the familiar Nernst formula for the P.D. arising from a diffusion gradient:

$$\text{P.D.} = \frac{u - v}{u + v} \frac{RT}{F} \ln \frac{\text{concn.}_1}{\text{concn.}_2}$$

This formula has been derived by Riesenfeld[16] for chains in which aqueous solutions containing the same electrolyte at different concentrations are separated by a solution of the electrolyte in a non-aqueous solvent immiscible with water, assuming that the ionic distribution ratios are equal at the two phase boundaries, and hence that the P.D.'s at the two boundaries are equal and opposite. In this case, u and v represent the mobilities of the cation and anion in the non-aqueous phase. Michaelis[17] has employed this equation for calculating transfer numbers in molecular seive membranes.

If this expression holds also for the sea water concentration effect in *Valonia*, *i.e.*, if the factor, $\frac{3}{5}$, in equation (1) represents the difference between the transfer numbers of anion and cation, we may calculate the transfer numbers in the protoplasm (or its external surface layer) assuming that a single ion pair is responsible for the P.D. Since the

[14] Höber, R., and Höber, J., *Arch. ges. Physiol.*, 1928, **219**, 260. Höber, R., and Hoffmann, F., *ibid.*, 1928, **220**, 558.

[15] *Cf.* Cooper, W. C., Jr., Dorcas, M. J., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1928–29, **12**, 427.

[16] See reference to Riesenfeld and Reinhold, *Z. physik. Chem.*, 1909, **68**, 459, in the chapter by Cremer, M., *Handb. norm. u. path. Physiol.*, 1928, **8**, 1003–4.

[17] Michaelis, L., Molecular sieve membranes, in Molecular physics in relation to biology, *Bulletin National Research Council*, 1929, No. 69, pp. 119–145.

more concentrated solution is positive, and hence the mobility of the anion is greater than that of the cation, we set:

$$\frac{v - u}{v + u} = \frac{2}{3}$$

and find $v = 5u$, *i.e.*, that the mobility of the anion is five times as great as that of the cation. The transfer number of the anion, $\frac{v}{u + v}$, is then 0.83, and that of the cation, $\frac{u}{u + v}$, is 0.17. The anion and cation in question are apparently Cl^- and Na^+.

It can be shown experimentally that changes in P.D. produced by dilution of the external sea water are due principally to changes in concentration of Na^+ and Cl^-, the changes in concentration of other ions in sea water playing a relatively small part. Attempts to measure the concentration effect with solutions of pure NaCl indicate that the more concentrated solution is positive, as with sea water.

Accurate measurements are impracticable, however, since fluctuations in the P.D. show that the system is changing continuously. Evidently the protoplasm is altered by contact with such unbalanced solutions. We can demonstrate the effect of the concentrations of Na^+ and Cl^- in another way, however. We compare the P.D. observed (1) with diluted sea water, in which the concentrations of all salts are reduced by the same factor, and (2) with an artificial sea water in which the concentrations of Na^+ and Cl^- alone are reduced, the other principal constituents remaining approximately the same as in undiluted sea water.

In Fig. 3 are shown graphically the results of such an experiment, comparing the P.D.'s with 0.33 natural and artificial sea water and

the P.D. with what we may call $Na^+/_3$, $Cl^-/_3$ sea water,[18] in which Na^+ and Cl^- have the same concentration as in the 0.33 sea water, while the other principal constituents of sea water, except $SO_4^=$, remain approximately unchanged. (An excess of sulfate is added to replace a part of the chloride, since the decrease in Cl^- is greater than in Na^+.)[18] It will be noted that the effect of changing the concentrations of Na^+ and Cl^- alone is even greater than that of diluting all the salts. The discrepancy is not large, however: after the P.D. with 0.33 sea water has reached its full value, all readings with all three solutions lie within the range 23.8 ± 0.8 mv. This is in good agreement with the value, 23.6 mv., calculated by adding $\frac{2}{3}\frac{RT}{F}$ ln 3 to the 5.0 mv., observed with sea water (temperature, 21°C.).

[18] The following table gives the composition of the $Na^+/_3$, $Cl^-/_3$ sea water, and, for comparison, the formula for artificial sea water on which it is based. This is a simplified form of the formula recommended by McClendon, Gault, and Mulholland, *Publ. No. 251, Carnegie Inst. of Washington* (1917). It will be noted that the $Na^+/_3$, $Cl^-/_3$ sea water contains an excess of sulfate, added to replace a part of the chloride, since the decrease in Cl^- is greater than in Na^+. Glycerol is added to make the solution isotonic with sea water.

	Artificial sea water	$Na^+/_3$, $Cl^-/_3$ sea water
	cc.	*cc.*
Normal solutions of $CaCl_2$	22.0	22.0
$MgCl_2$	50.4	
$MgSO_4$	57.2	107.6
KCl	10.2	10.2
NaCl	483.6	156.5
NaBr	0.8	0.8
$NaHCO_3$	2.5	2.5
Na_2SO_4		2.5
8.7 per cent solution of glycerol		550.0
Distilled water to make	1000.0	1000.0
Total chlorides	566.2	188.7
Total sodium salts	486.9	162.3

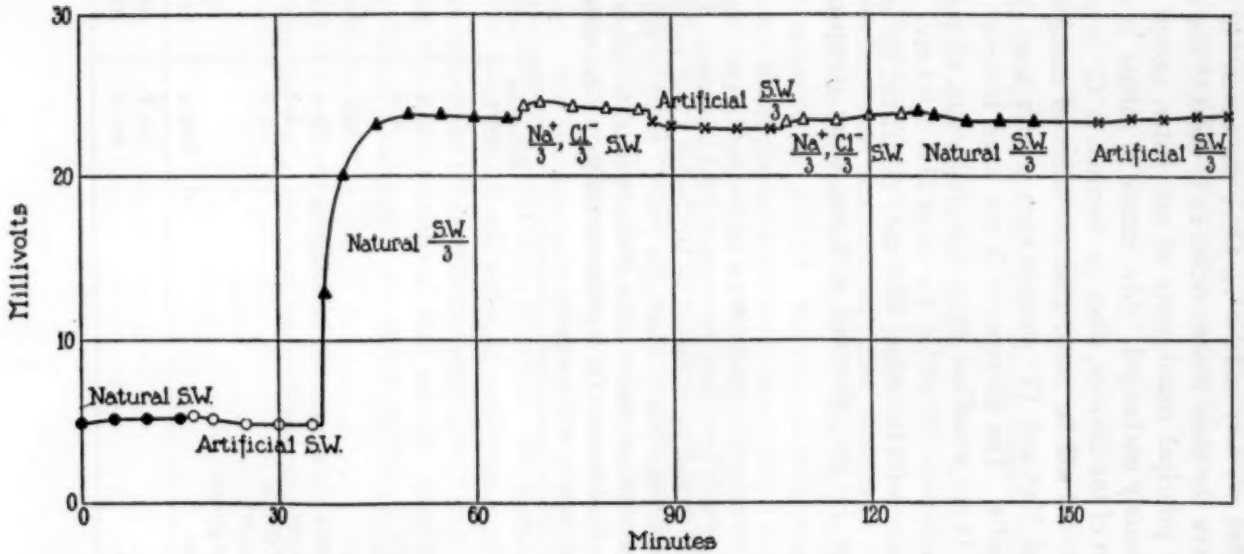

FIG. 3. P.D.-time curve, comparing the P.D.'s with 0.33 (natural and artificial) sea water and that with an artificial solution in which the concentrations of Na^+ and Cl^- are approximately the same as in 0.33 sea water, while the concentrations of the other principal ions of sea water are the same as in ordinary sea water, or somewhat greater ($SO_4^=$).

SUMMARY

The P.D. between the interior of a cell of *Valonia macrophysa* and concentrated or diluted sea water applied externally is given by the empirical equation:

$$\text{P.D.} = \text{P.D.}_{\text{s.w.}} + \frac{2}{3}\frac{RT}{F}\ln\frac{1}{\text{concn.}}$$

where $\text{P.D.}_{\text{s.w.}}$ is the value observed when the external solution is sea water. The sign is that of the interior of the cell. In the chain:

sea water, concn._1 | *Valonia* | sea water, concn._2

therefore, the more concentrated solution is positive in the external circuit to the more dilute solution.

This holds for the concentration range 1.5 sea water to 0.2 sea water, when the dilute solutions are made isotonic with sea water by the addition of a suitable non-electrolyte (such as glycerol). Prolonged exposure to these solutions, or brief exposure to very concentrated or very dilute sea water, or to hypotonic solutions, produces in the cell secondary changes which are made evident by the reversal of the sign of the P.D. These changes are to a certain extent reversible, but if allowed to proceed too far they become irreversible, involving permanent injury to the cell.

The concentration effect with sea water is shown to be practically the concentration effect for NaCl, the part played by the other salts in sea water being relatively small.

NEGATIVE VARIATIONS IN NITELLA PRODUCED BY CHLOROFORM AND BY POTASSIUM CHLORIDE

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(Accepted for publication, January 14, 1930)

Experiments by Mr. Harris in 1922 (in collaboration with the senior author) showed that chemical stimulation could set up negative variations in *Nitella*. Owing to the theoretical interest of this result an extensive series of experiments has been carried on since that time. The present paper deals with the effects of chloroform and KCl.

The technique, unless otherwise stated, is that described in previous papers.[1] The experiments were performed on *Nitella flexilis* at a temperature of 19° to 20°C. It was found advisable to keep each cell in a separate dish after cutting (to avoid the effects of sap coming from injured cells) and to let the cells stand for some hours or days after cutting. In placing the cells in the apparatus they were handled with extreme care to prevent as far as possible any mechanical disturbance.

In order to avoid stimulation by static discharge glass-tipped forceps were used, the operator standing on a grounded piece of copper. To avoid stimulation by mechanical shock the cotton saturated with solution was placed very gently on the cell or flowing contacts were used (that these precautions were effective is shown by the fact that applying cotton soaked in 0.001 M KCl did not start a variation).

In applying an E.M.F. between *A* and *C* the P.D. at *C* was measured by killing *A* with chloroform (this brings its P.D. aproximately to zero). In some cases the chloroform was applied before impressing the E.M.F. and in other cases afterward (in these latter cases a whole series of E.M.F.'s was applied in turn to be sure of getting the right value).

To prevent unintentional stimulation the time signal and the stimulus were completely insulated from each other and the stimulating circuit was closed by a platinum wire dipping into a mercury cup (to avoid an unintentional break in the circuit).

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[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673; 1928–29, **12**, 167.

According to the local circuit theory of stimulation[2] we should expect an application of chloroform to a cell imbibed with tap water or with 0.001 M KCl (*e.g.*, to such a point as *A*, Fig. 1) to start a negative variation for the following reason. As previous studies[3] have shown

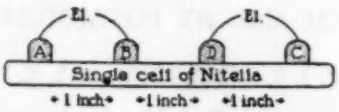

FIG. 1. Diagram to show arrangement of experiments

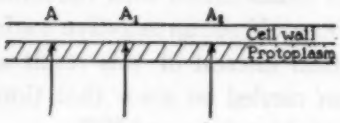

FIG. 2

FIG. 2. Hypothetical diagram of P.D.'s in the protoplasm when the cell wall is imbibed with tap water. The arrows show the direction in which the positive current tends to flow and their length indicates the relative magnitude.

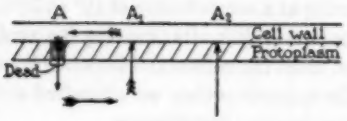

FIG. 3

FIG. 3. As in Fig. 2 but with the protoplasm at *A* shaded to indicate that it has been killed by chloroform. The feathered arrows denote flow of current, the plain arrow represents P.D. (it being assumed, as a convenient fiction, that there is no flow as yet at A_2).

FIG. 4

FIG. 4. As in Fig. 3 but with a flow between A_2 and A_1 (it being assumed, as a convenient fiction, that there is no flow at *A*). The protoplasm at *A* is shaded vertically to indicate that it has been killed by chloroform and that at A_1 shaded horizontally to indicate that it has temporarily lost its E.M.F.

that the P.D. across the protoplasm may be represented as in Fig. 2 and that when 0.001 M KCl saturated with chloroform is applied at *A* the

[2] *Cf.* Lillie, R. S., Protoplasmic action and nervous action, University of Chicago Press, Chicago, 1923. Davis, H., The condition of the nerve impulse, *Physiol. Rev.*, 1926, **6**, 547.

[3] *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673.

P.D. at that point rapidly approaches zero[3] we suppose that a current, due to the E.M.F. at A_1, flows between A_1 and A as shown in Fig. 3,

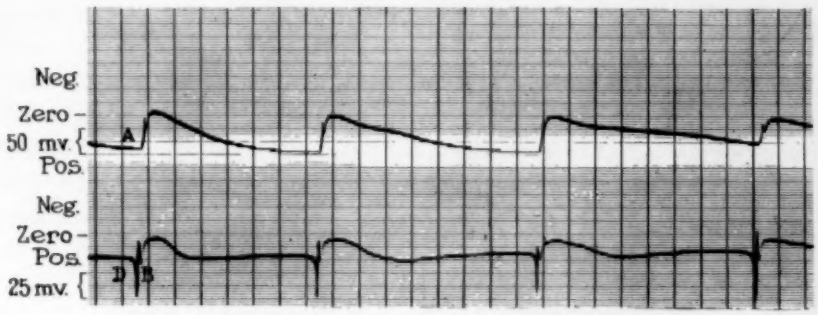

FIG. 5. Photographic record of successive variations produced by application of 0.001 M KCl saturated with chloroform at C (Fig. 5 *a*) with 0.001 M KCl at A, B and D. The two strings of the double string Einthoven galvanometer were connected as shown in Fig. 5 *a*.

A variation starting at C strikes D, making the downward movement labelled D in the record (this is really a negative change at D but appears positive because we record only the P.D. of B with reference to D). When the variation reaches B it makes the upward movement labelled B in the record. The P.D. then approaches zero. The successive variations starting from C produce similar effects.

When the variation reaches A it causes the upward movement labelled A in the record (*i.e.*, a negative variation at A): the variation is monophasic because the P.D. at C remains constant (approximately zero), the protoplasm having been killed by chloroform.

The vertical lines mark 5-second intervals.

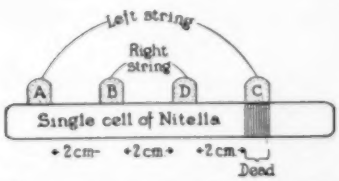

FIG. 5 *a*. Diagram to show arrangement of the experiment recorded in Fig. 5. Protoplasm shaded at C to show that it has been killed by chloroform. The left string gives the upper record in Fig. 5.

which quickly reduces the E.M.F. at A_1 approximately to zero (that it really goes almost or quite to zero is evident when we lead off from A_1

to a spot whose P.D. is zero).[4] We suppose that a flow then starts between A_1 and A_2 (Fig. 4) and thus the variation travels along the cell.[5] The P.D. at A_1 and A_2 soon returns to normal and the whole process can commence anew. Thus successive disturbances can be produced as shown in Fig. 5 (in some cases a dozen or more).

The interval between successive disturbances depends upon the refractory period which follows each stimulation. It has been found by Blinks[6] that when the E.M.F. approaches zero, as the result of the outward flow of positive current, the resistance (as measured by direct current) falls almost or quite to that of a dead spot. There is therefore a period during which cations are moved outward by the current: as

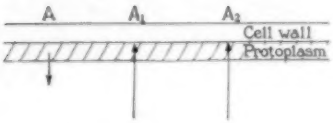

FIG. 6. As in Fig. 2 but with 0.05 M KCl at A in place of chloroform.

a result they may increase in concentration just outside the outer protoplasmic surface. This must decrease the E.M.F. across the protoplasm until such cations are removed by outward diffusion[7] or by reabsorption into the cell aided by the inward current which follows the outward current. This may explain the refractory period after each stimulus and the increasing fatigue following repeated stimuli, but it is possible that chemical or structural changes, such as those suggested by Osterhout and Harris,[3] may also play a part.

We should expect similar results if 0.05 M KCl (without chloroform) were applied at A since this would usually give to the arrow at A the direction shown in Fig. 6[3] which should produce a greater flow of cur-

[4] This will be dealt with in other papers: it does not go all the way to zero in all cases since the all or none law does not always strictly apply.

[5] The variation travels in both directions from A but for convenience only one side is here represented.

[6] Blinks, L. R., Harris, E. S., and Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 836. Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 495.

[7] This diffusion away from the outer surface can readily occur since, relatively speaking, the actual quantity of cations coming out is exceedingly small. The external solution as a whole therefore remains practically constant as shown by the potentials after successive recoveries.

rent than the application of chloroform. This expectation seems to be realized since it is a simple matter to produce a succession of disturbances by 0.05 M KCl (Fig. 7).

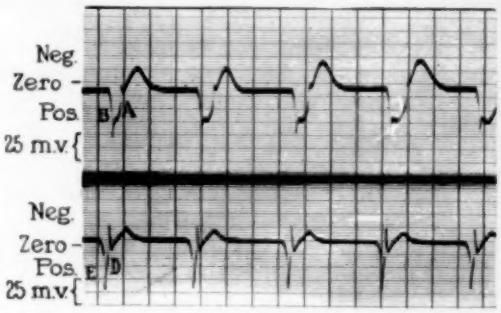

FIG. 7. Photographic record showing successive variations due to application of 0.05 M KCl at *C* (Fig. 7 *a*), with 0.001 M KCl at *A*, *B*, *D*, and *E*. The two strings of the double string Einthoven galvanometer were connected as shown in Fig. 7 *a*.

A variation starting at *C* strikes *E* making the downward movement labelled *E* in the record (this is really a negative change at *E* but appears positive because we record only the P.D. of *D* with reference to *E*). When the variation reaches *D* it makes the upward movement labelled *D* in the record. The P.D. then approaches zero. The successive variations starting from *C* produce similar effects.

When the variation reaches *B* it causes the downward movement labelled *B* in the record (this is really a negative change at *B* but appears positive because we record only the P.D. of *A* with reference to *B*). When the variation reaches *A* it makes the upward movement labelled *A* in the record.

The vertical lines mark 5-second intervals.

The fact that the refractory period is shorter than in Fig. 5 apparently has no relation to the stimulus in this case.

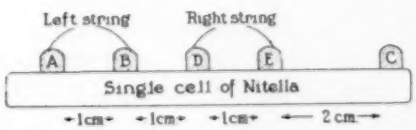

FIG. 7 *a*. Diagram to show arrangement of the experiment recorded in Fig. 7. The left string gives the upper record in Fig. 7.

With lower concentrations the results are different: for example, 0.01 M and 0.02 M KCl usually produce only one wave. This is to be

expected if we consider that a wave can start only when a sufficient P.D. gradient exists: thus in Fig. 8 the P.D. gradient G_1 might be sufficient to start a variation because the flow of current through A_3 could break down its P.D. whereas this might not happen with the P.D. gradient G when the flow through A_1 might be insufficient.

These P.D. gradients will depend on the diffusion gradients: this is illustrated in Fig. 9 which shows the effect of the diffusion gradient

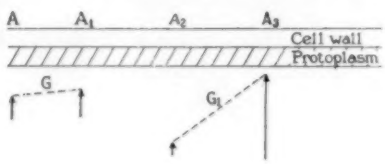

FIG. 8. Hypothetical diagram illustrating a gentle gradient of P.D. (G) and a steep one (G_1).

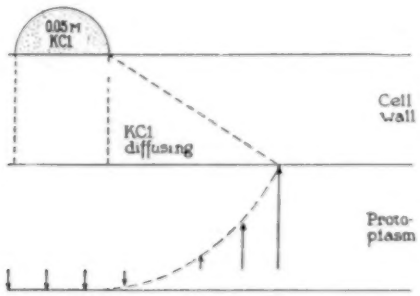

FIG. 9. Hypothetical diagram showing the effect of 0.05 M KCl (diffusing along the cell wall) upon the P.D. gradient in the protoplasm. The concentration of KCl in the cell wall is denoted by the height of the broken line in the cell wall.

on the P.D. gradient. It is evident that the higher the concentration[8] of applied KCl the steeper will be the diffusion gradient (with equal times of diffusion). The longer the time elapsing since the application the less steep will be the diffusion gradient and the smaller the chance of starting a negative variation. Hence it is clear that as we lower the concentration we shorten the time during which the necessary steepness of gradient will persist and finally a concentration will be reached where

[8] For example, excellent results are obtained with 0.1 M KCl.

only one disturbance will occur: at still lower concentrations none will start.

In the same way chloroform (or any substance produced by its action) diffusing along the cell might lower the P.D. gradient which may explain why we often get only a few negative variations (sometimes only one or none at all).

If chloroform or 0.05 M KCl does not produce a sufficiently steep gradient to start a variation we may easily remedy the trouble by

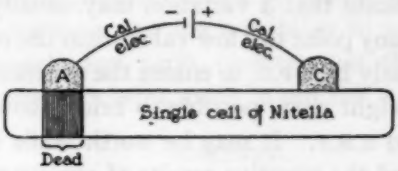

FIG. 10. Diagram to show the method of imposing E.M.F. between *A* and *C*. The protoplasm at *A* is shaded to show that it has been killed by chloroform.

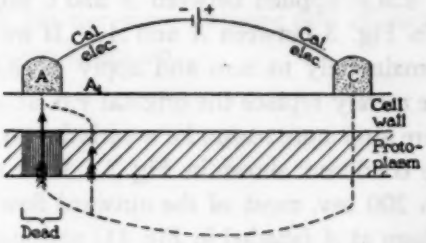

FIG. 11. Diagram to show flow of current when an E.M.F. is imposed between *A* and *C*: the flow is greater at *A* than at A_1. The protoplasm at *A* is shaded to show that it has been killed by chloroform.

running a wire from the calomel electrode at the spot in question to the calomel electrode at a spot some distance away where the protoplasm is in contact with 0.001 M KCl and hence has a high P.D. This gives a sufficiently high gradient and a variation starts at once at the distant point.

When 0.05 M or 0.01 M KCl has stopped producing variations we can usually start them by moving the wad of cotton to a fresh spot, thus starting a new gradient.

A variation cannot pass a spot to which 0.001 M KCl saturated with

chloroform has been applied nor, as a rule, one which is in contact with 0.01 M KCl or 0.05 M KCl. This is to be expected since all of these agents either reduce the P.D. to a low value or reverse its direction.

We may therefore use these substances to block the passage of a variation: very convenient for this purpose is 0.02 M KCl which very seldom starts more than one; for still greater certainty we may use a piece of cotton saturated with 0.05 M KCl on each side of which we place cotton saturated with 0.02 M KCl.

These results indicate that a variation may usually be started by bringing the P.D. at any point to a low value when the rest of the protoplasm has a sufficiently high P.D. to ensure the necessary outward flow of current, and it might seem possible to bring about this result by means of an applied E.M.F. It may be worth while to consider this briefly in the light of the negative results of our experiments on this point.

If the cell is in the state shown in Fig. 2 (with each arrow representing 100 mv.) an E.M.F. applied between A and C might start a flow such as is seen in Fig. 3 between A and A_1. If we first reduce the E.M.F. at A approximately to zero and apply an E.M.F. of 100 mv. (as in Fig. 10) we merely replace the original P.D. at A by the applied E.M.F. so that there is no more reason for a variation to start than when the cell is in the condition[9] shown in Fig. 2. If we now increase the applied E.M.F. to 200 mv. most of the outward flow will be through the dead protoplasm at A (shaded in Fig. 11) and much less will flow through A_1 (where the resistance is much higher): what flows through A will not start a variation but what flows through A_1 may do so if the flow is sufficient.

A variation coming down the cell and reaching such a point as C in Fig. 10 may not be able to pass since the applied E.M.F. tends to form an inward current (by an inward current is meant one like that shown by the arrow at A in Fig. 3): this, according to unpublished experiments by Dr. Blinks, must reach a relatively high value before it can reduce the E.M.F. of the protoplasm to zero ("anodal stimulation") but this can be done by a relatively small outward current

[9] The chief difference is that A and C are connected by a wire as well as by the cell wall but this cannot start a variation unless sufficient difference of P.D. exists between them.

(*i.e.*, a current like that shown at A_1 in Fig. 3) and this is what happens when a negative variation travels along the cell, *i.e.*, each spot which is traversed by an outward current loses its E.M.F. in turn. If such outward currents are sufficiently reduced by an opposing E.M.F. as at *C* in Fig. 10 the variation cannot pass. This is an "anodal block."

The successive variations in *Nitella* recall those observed in the heart and in certain cases in nerve (since in nerve there is no contraction the analogy may be closer). In all these cases the action currents are similar in form and magnitude but in *Nitella* the process is much slower. These facts suggest the possibility of producing successive variations in muscle and nerve in somewhat the same way as in *Nitella*: experiments are being made to test this suggestion.

SUMMARY

The results of applying chloroform and KCl to *Nitella* indicate that a negative variation may be started whenever it is possible to set up along the protoplasm a gradient of potential difference sufficiently steep to produce the necessary outward flow of current. Successive variations may thus be set up.

PROTEIN COAGULATION AND ITS REVERSAL

The Preparation of Insoluble Globin, Soluble Globin and Heme

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The Reversibility of Coagulation

It has already been shown that the coagulation of hemoglobin appears to be reversible. One can prepare from completely coagulated hemoglobin soluble, crystalline hemoglobin which by the tests so far tried is indistinguishable from ordinary native hemoglobin (Mirsky and Anson (13)). Since hemoglobin is a typical coagulable protein (Anson and Mirsky (3)) the conclusion was drawn that protein coagulation in general is probably reversible. In support of this view there is now the fact that one can prepare from globin coagulated by heat or by acid acetone a soluble globin which can be coagulated again by heat and which can combine with heme to form crystallizable hemoglobin. Whether or not this soluble globin is identical with native globin as it exists in hemoglobin is not yet decided. The present paper describes empirically the technique for preparing globin. A later paper will deal with the crystallization of synthetic hemoglobin, the preparation of soluble globin from heat coagulated globin, the factors determining the apparent reversibility of protein denaturation, the temperature of coagulation and the thiol groups of globin and the precipitation of insoluble globin in the presence of soluble globin.

The Preparation of Globin

The Present Procedure.—The method for preparing soluble globin now to be described involves two separate steps. In the *first step*

acid acetone is added to an acid solution of carbon monoxide hemoglobin whereupon the hemoglobin is separated into a solution of heme and a practically pigment-free precipitate of denatured globin combined with hydrochloric acid. After removal of the acetone the globin powder readily dissolves in water to give an acid solution. The globin may be completely precipitated from this acid solution by third saturation with ammonium sulphate and almost completely precipitated by rapid complete neutralization. In the *second step* of the preparation, the acid solution of the globin powder is gradually neutralized by one of several special procedures which avoid rapid complete neutralization. Thereupon about 65 per cent of the horse globin and about 80 per cent of ox globin are converted into a form which is soluble in a neutral solution 0.4 saturated with ammonium sulfate and which can combine with heme to form hemoglobin.

Historical.—Before describing in detail the preparation which has just been outlined, we shall review the older methods for obtaining globin and heme.

In 1898 Schulz made the first study of the properties of a fairly pure globin prepared by adding acid to hemoglobin and extracting the pigment with alcohol and ether. Schulz's globin was insoluble around pH 8 but readily soluble in acid or alkali. Due to the peculiar properties discovered by Schulz, globin was classified with the histones.

In 1925 it was pointed out (1) that the classical globin of Schulz is not the native globin of hemoglobin but a denatured protein, like other denatured proteins insoluble at its isoelectric point, and (2) that while hemoglobin itself is a compound of native globin and heme, the hemochromogen prepared from hemoglobin is a compound of denatured globin and heme (Anson and Mirsky (2)).

These views were confirmed by Hill and Holden (9) who then made the new and important contribution of preparing for the first time soluble, apparently native globin which can combine with heme to form hemoglobin. It will be shown in another paper that Hill and Holden's theory of the preparation of native globin has no experimental basis and that the preparation based on their theory gives almost the lowest possible yield, only by accident any appreciable yield at all.

The synthesis of hemoglobin, in itself, was not new. Bertin-Sans and de Moitessier had already synthesized hemoglobin from pigment-free globin in 1893 (4). The writers (1) likewise had prepared hemoglobin from Schulz's globin before they realized that denaturation of the protein is involved in Schulz's procedure. Holden and Freeman (10) who repeated and confirmed our experiments have suggested, perhaps correctly, that Schulz's globin although mainly denatured

and insoluble, nevertheless contains some soluble globin and that it was this soluble fraction which was the source of our synthetic hemoglobin.

Holden and Freeman (10) have also improved the technique for the preparation of soluble globin introduced by Hill and Holden. They stated that they paid especial attention to the yield. They did not state what the yield was.

Wu (17) found that Hill and Holden's method gave a yield of only a few per cent. He prepared soluble globin by simply carrying out Schulz's procedure in the cold.

It remains to describe the old acid acetone preparation of heme (Merunowicz and Zaleski (12), Hamsik (7)) which forms the basis for the present preparation of insoluble globin. The procedure consists simply in a slow extraction of coagulated hemoglobin with acid acetone. It yields a protein-free solution of heme without the use of the high temperature or strong acid required by the classical glacial acetic acid technique. It did not in its previous form yield a globin reasonably free of pigment and so was not used for the preparation of globin. The present modification gives an immediate and almost complete separation of heme and globin.

Preparation of Hemoglobin

Ox corpuscles washed four times with isotonic salt solution are shaken with an equal volume of alumina cream (Tracy and Welker (15)) and a quarter of the total volume of toluol (Heidelberger (8)) and allowed to stand in the cold. The next morning the clear solution on bottom is siphoned off and filtered. The suspension of stromata is centrifuged and the clear solution on bottom is filtered. In the case of horse blood it is desirable to add 10 per cent more alumina cream after adding water to the corpuscles to the extent of 10 per cent of their volume. This greater dilution is to prevent the crystallization of the less soluble horse hemoglobin. Unfortunately the alumina cream used in these preparations did not have the property described by Tracy and Welker of precipitating quantitatively the serum proteins but it proved useful, nevertheless, in facilitating the rapid and complete separation of stromata and toluol.

Preparation of Insoluble Globin Powder and Heme Solution

For each 10 cc. of hemoglobin solution cooled in ice water are added 10 cc. of cold 0.1 N HCl and 200 cc. of acetone (not cooled) containing 2 cc. of 1 N HCl. The suspension is thoroughly shaken and filtered with gentle suction. The filtrate, which is saved for the preparation of heme, gives no further precipitate on the addition of acetone, dilute sodium acetate or trichloracetic acid. While the globin is still on the filter paper it is washed with acetone and drained with full suction. It is then pressed between filter papers, removed and dried in the air. It loses 5 per cent of its weight on being dried further at 100°. This dry acid globin is practically free of pigment, is readily dissolved in water to give a clear

solution, and is completely precipitated from such a solution when it is one-third saturated with ammonium sulphate.

If the slightly pigmented globin is dissolved in alkali and the reducer $Na_2S_2O_4$ added, a small amount of hemochromogen is obtained whose α band (in the case of horse globin) is about 10 Å. to the blue of the α band of the globin hemochromogen prepared from hemoglobin, hemin or acid acetone heme. In other words, the small amount of pigment which remains attached to the globin is in part at least a modified heme. If oxalic acid is used instead of HCl in the preparation of the globin then the globin contains still less pigment and the globin hemochromogen this pigment yields has its α band in the normal position. This oxalic acid globin, however, is less useful for the preparation of soluble globin than is the HCl globin. The procedure with oxalic acid is simply to add to a 5 per cent solution of carbon monoxide hemoglobin ten times its volume of acetone containing 2.5 gm. oxalic acid per 100 cc. acetone.

Preparation of Soluble Globin

When an acid solution of the acetone globin is neutralized part of the protein is precipitated and part remains in solution. On 0.4 saturation of the neutral filtrate with ammonium sulfate a small additional precipitate is obtained. The insoluble fraction has the characteristics of denatured protein; it is insoluble at its isoelectric point (about pH 8) and it combines with reduced heme to form hemochromogen. The fraction soluble in 0.4 saturated ammonium sulfate has the properties one would expect native globin to have; it is soluble in distilled water, it can be salted out by seven-tenths saturation with ammonium sulfate and then redissolved by the addition of a little water, it can be coagulated by heat or by shaking, and it can combine with heme to give a substance with the spectrum of methemoglobin. The methemoglobin may be reduced with $Na_2S_2O_4$ to give a pigment with the typical spectrum of reduced hemoglobin. When a solution of this reduced hemoglobin is shaken with air there appears the spectrum of oxyhemoglobin, the α band being in the normal position, within the experimental error of 2 Å. Whether the soluble globin is *identical* with globin as it exists in hemoglobin is not decided by the present experiments.* It is likewise not proven that some insoluble

* In solubility experiments normal hemoglobin and hemoglobin synthesized from soluble globin behave as if they were one and the same component in the sense of Willard Gibbs.

globin is not kept in solution by soluble globin even in 0.4 saturated ammonium sulfate.

Factors Influencing the Yield.—What fraction of the globin is precipitated on neutralization and 0.4 saturation with ammonium sulfate and what fraction is obtained in the form of soluble, apparently native globin depends primarily on how the neutralization is carried out. If the neutralization is rapid and complete, practically all the globin is precipitated. If the alkali is added in two steps, first just not enough to cause turbidity and after an interval of time, the rest, then most of the globin remains in solution. Similarly a high yield of soluble globin is obtained if most of the acid is first removed gradually by dialysis.

A variety of neutralization experiments together with a discussion of their bearing on the question of what factors influence of the apparent reversal of denaturation will be presented in a following paper. The purpose of the present paper is merely to describe empirically a technique for obtaining soluble globin.

Neutralization Method.—To a 5 per cent solution in water of the acetone globin powder there is gradually added with shaking an amount of 0.1 N NaOH which is 95 per cent of that required to give the first permanent turbidity. Then, after ten minutes, enough more 0.1 N NaOH is added to give the maximum precipitation. The amounts of NaOH required are determined empirically for each batch of globin. In the case of one sample of horse globin to each 100 cc. of 5 per cent solution there were added first 50 cc. 0.1 N NaOH and then 12.5 cc.

The neutralized solution of globin is now 0.4 saturated with ammonium sulfate to precipitate the insoluble globin as completely as possible and is filtered. The globin left in solution is precipitated by the addition of 16 gm. solid ammonium sulfate for each 100 cc. of solution. After filtration the precipitate is put in collodion membranes and dialyzed overnight at 5°C. in a shaking dialyzer (Kunitz and Simms (11)). Thus a concentrated salt-free solution is obtained.

Yield.—The yield is conveniently determined by estimation of the globin concentration in the solution which is 0.4 saturated with ammonium sulfate. To 10 cc. of a diluted solution containing about 3 mg. of protein are added 2 cc. of 20 per cent trichloracetic acid. After centrifugation and rejection of the supernatant fluid, the precipitate is dissolved in 2 cc. 0.1 N NaOH and estimated by means of the color developed by the phenol reagent of Folin and Ciocalteau (5), in essentially the manner first described by Wu (16). The modifica-

tions of the directions of Wu which have been used in this laboratory for several years for a number of proteins are practically the same as the modifications recently published by Greenberg (6). As a standard the original solution of the acetone globin powder is used. This is justified because both the insoluble and soluble forms of globin after precipitation with trichloracetic acid have the same color value per milligram of nitrogen. The ammonium sulfate which remains with the trichloracetic acid precipitate has no influence on the color.

Yield by the neutralization method: Horse globin, 65 per cent.

Dialysis Method.—A 2.5 per cent solution of the acetone powder is dialyzed overnight at 5°C. against distilled water in a shaking dialyzer. No precipitate is formed because the solution is not completely neutralized by the dialysis. The insoluble globin may be precipitated by the addition of an equal volume of a $K_2HPO_4-(NH_4)_2SO_4$ solution containing one part 1 M K_2HPO_4 to five parts saturated ammonium sulfate. Or the solution may be neutralized by dialysis against M/30 K_2HPO_4. When the precipitate formed is filtered off, 0.4 saturation of the filtrate with ammonium sulfate causes only a faint haze to appear.

Yields: Horse globin, 35 per cent; ox globin, 80 per cent.

Preparation of Heme

There is available a number of ways of precipitating heme from the acid acetone solution, removing the acetone which remains with the precipitate and redissolving the acetone-free heme without the use of strong alkali which may modify the heme.

Water Precipitation.—The heme is precipitated by the addition to the acid acetone solution of twice its volume of water. After filtration the heme precipitate is washed on the filter paper with water and dissolved while still fresh by the addition of 1 M K_2HPO_4.

NaAc Precipitation.—Much more convenient is the precipitation by addition to the acid acetone solution of 1 per cent its volume of 2 N sodium acetate. There is less solution to filter than when water is added, the filtration is faster, and the acetone may readily be recovered from the filtrate by distillation after removal of the water with $CaCl_2$.

The NaAc precipitate may be washed with 1/15 M KH_2PO_4 and then dissolved in a buffer consisting of equal parts of Na_2CO_3 and $NaHCO_3$. Or the precipitate may be dissolved in acetone phosphate solution by adding 0.5 M K_2HPO_4 to a suspension of heme in acetone and then diluting with water. The acetone may be removed by vacuum distillation or if the acetone concentration is not too high, by

dialysis against any slightly alkaline buffer such as K_2HPO_4. In slightly alkaline solution heme does not pass through collodion membranes made to retain hemoglobin. Apparently under these conditions heme exists not in the form of molecules containing one iron atom and having a molecular weight of 650 but in the form of aggregates. An alternative procedure for removing the acetone from the acetone phosphate solution is to precipitate the heme with HCl, wash with water and redissolve in K_2HPO_4.

If it is desired to store the heme indefinitely, the NaAc precipitate may be washed with acetone and dried in the air. The powder dissolves slowly in 0.1 N NaOH, and not at all in K_2HPO_4. It may be dissolved slowly in the acetone phosphate solution.

The precise directions for the preparation of the acid acetone solution were worked out in order to get the best separation of globin and pigment. If only the heme is desired, it is not essential to use CO or low temperature and the procedure may be modified to require less acetone. Economy of acetone, however, is not important since the acetone may readily be recovered.

The properties of the acid acetone heme have not as yet been investigated. In particular, it has not been determined whether the heme dissolved in K_2HPO_4 is identical with the heme dissolved in NaOH and then neutralized with KH_2PO_4.

SUMMARY

1. By a procedure involving the use of acid acetone hemoglobin may be rapidly separated into a precipitate of denatured globin and an acetone solution of heme.

2. By neutralization procedures the denatured globin may be largely converted into a soluble, apparently native form which can combine with heme to form hemoglobin.

3. The heme may be obtained in acetone-free, slightly alkaline solution without the use of strong alkali which may modify the heme.

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PROTEIN COAGULATION AND ITS REVERSAL

Improved Methods for the Reversal of the Coagulation of Hemoglobin

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The coagulation of proteins takes place in two steps. The first step is denaturation, a change in the protein which makes it insoluble at the isoelectric point, although denatured protein is still soluble in acid or alkali. The second step is simply the precipitation of the insoluble denatured protein. It is usually supposed that denaturation is irreversible. We have already described experiments, however, which indicate that the denaturation and coagulation of hemoglobin, a typical coagulable protein, are reversible (1, 4). From hemoglobin completely coagulated by heat, acid or urea it was possible to prepare, with a yield of about 30 per cent, soluble crystalline hemoglobin which by the tests so far tried has been indistinguishable from normal native hemoglobin. The procedures for apparent reversal and preparing crystals of "reversed" hemoglobin have now been improved so that they are simpler and quicker and give yields of 70 to 75 per cent. Furthermore, the soluble crystalline hemoglobin has now been prepared from hemoglobin coagulated by shaking* and trichloracetic acid as well as by heat, acid, and urea. Horse hemoglobin has been used in the present experiments because it is readily crystallizable. Exactly the same experiments may be done with ox hemoglobin, the yields of soluble protein being in general higher than with horse hemoglobin.

The technique for reversal although modified has not been changed essentially. It consists as before mainly in bringing an acid solution

* The surface coagulation of hemoglobin and the reversal of surface coagulation will be discussed in a separate paper.

of denatured hemoglobin close to the isoelectric point but not close enough to cause precipitation. Previously the denatured hemoglobin was brought into a slightly alkaline solution containing cyanide, the use of which seemed necessary for an adequate yield. This same procedure has been retained with, however, much less cyanide in the solution. And the reversal has been accomplished from both the acid and the alkaline sides without the use of any cyanide at all.

Denaturation by HCl.—A 10 per cent solution of horse carbon monoxide hemoglobin is prepared as already described (2). To such a solution at 0° is added an equal volume of 1/15 N HCl. After 3 minutes the acid solution is used for the various reversal procedures. There is evidence that this time suffices for complete denaturation. If the acid solution is rapidly and completely neutralized with NaOH after 3 minutes, then practically all the protein is precipitated, which would not happen were any soluble native hemoglobin present. Furthermore, if the reversal procedure is begun 18 hours after the addition of the acid then the same 70 to 75 per cent yield of soluble hemoglobin is obtained as when the reversal procedure is begun after 3 minutes. Either there is no further denaturation after 3 minutes or hemoglobin cannot be denatured more than 25 per cent under the conditions of the experiment. It might be supposed that all the denaturation is due to local excesses of HCl during the addition of the acid, that no further denaturation takes place after the acid is mixed with the solution, and that there is accordingly no further denaturation with time beyond the 25 per cent which supposedly takes place during the mixing. This hypothesis is disproved by two facts. First, if the solution is neutralized *immediately* after the addition of acid, then a great deal of protein is not precipitated, showing that there must be some change after the addition of acid. Secondly, the yield is the same regardless of the manner of adding the acid, that is regardless of the extent of temporary local excesses of HCl.

Reversal by NaCN.—To 4 volumes of the acid solution of denatured hemoglobin which contains 3 volumes of 1/15 N HCl is added in the cold a mixture of 1 volume of 1/5 N NaOH and 1/20 volume of 1 N NaCN. No permanent precipitate is formed. After the solution has stood 1 hour at room temperature an equal volume of saturated ammonium sulfate is added and the resulting precipitate filtered off. The 75 per cent of the original protein which remains in solution as cyan methemoglobin may be converted into crystals of carbon monoxide hemoglobin and may be coagulated again.

Yield.—The fraction of the protein remaining in solution after half saturation with ammonium sulfate depends on how long the cyanide solution has stood before the addition of the ammonium sulfate. At room temperature the maximum yield of 70 to 75 per cent

is reached in 1 hour, at 0° in a day (see Table I). The fact that 90 per cent of the protein is precipitated if the ammonium sulfate is added immediately after neutralization and that it takes time to get the soluble form is further evidence that the soluble form did not already exist in the acid solution, that the protein was completely denatured by the acid.

A simple colorimetric method was used to obtain the yield, the hemoglobin being determined as so called alkaline cyan hematin. The method was first checked by Fe and N analyses. To 1 cc. of the ammonium sulfate filtrate is added 9 cc. water and, after mixing, 2 cc. of 20 per cent trichloracetic acid. The centrifuged precipitate is dissolved with 2 cc. of 1/5 N NaOH and 1 cc. of 1 N

TABLE I

Effect of Time and Temperature on Per Cent Yield on Reversal

Time in CN before addition of $(NH_4)_2SO_4$	Per cent not precipitated by half saturation with $(NH_4)_2SO_4$	
	0°	22°
1 minute	10	
10 minutes	34	64
30 "	48	72
1 hour	52	76
2 hours	54	78
5 "	62	78
21 "	69	80
65 "	75	76

KCN and diluted to 10 cc. The standard is made up from the original alkaline cyanide solution to which 0.5 cc. saturated ammonium sulfate is added after dilution to 9.5 cc.

Reversal by Neutralization.—It has already been stated that if the hemoglobin solution which has stood in 1/15 N HCl for 3 minutes is rapidly and completely neutralized by the addition of an equivalent amount of NaOH practically all the protein is precipitated. About 50 per cent of protein soluble in half saturated ammonium sulfate is obtained, however, if the neutralization is carried out in two steps, first just not enough to cause a permanent turbidity, then, after a time, the rest.

For instance, to 10 cc. of 10 per cent horse carbon monoxide hemoglobin is added 30 cc. 1/15 N HCl in the cold. After 3 minutes the solution is brought to room temperature and 15 cc. 1/10 N NaOH are added gradually. 2 hours later 5 cc. more 1/10 N NaOH are added to complete the neutralization and then an equal

volume of saturated ammonium sulfate. 51 per cent of the protein is precipitated. Precisely analogous experiments may be done with denatured globin as is described in another paper (2).

Instead of neutralizing the HCl, the acid may be removed by shaking dialysis in the cold. The yield is about 50 per cent. Again similar experiments may be done with globin (2).

Coagulation by Trichloracetic Acid.—To 10 cc. of 10 per cent HbCO are added 25 cc. H_2O and 10 cc. of 20 per cent trichloracetic acid. The suspension is centrifuged and the supernatant liquid titrated with NaOH. To the centrifuged precipitate suspended in 25 cc. of water there is now added a mixture of 0.5 cc. 1 N KCN and an amount of 1/5 N NaOH equivalent to the acid remaining with the precipitate. Solution takes place immediately. After 1½ hours 51 per cent of the protein is precipitated by half saturation with ammonium sulfate.

Crystallization.—The soluble cyan methemoglobins obtained from the hemoglobins denatured by HCl, trichloracetic acid, and shaking have all been converted into carbon monoxide hemoglobins and then into crystals of the normal form. For the purpose of merely demonstrating the crystallizability of the "reversed" hemoglobin, crystallization by ammonium sulfate is the simplest procedure. To get pure hemoglobin, the hemoglobin is concentrated by pressure dialysis of an isoelectric solution whereupon crystals come out of the supersaturated solution.

In either procedure the cyan methemoglobin is first precipitated and thus concentrated by the addition of 16 gm. solid ammonium sulfate to each 100 cc. of the solution already half saturated with ammonium sulfate. In the ammonium sulfate crystallization procedure a concentrated suspension of the filtered precipitate is now dissolved in a minimum amount of water, the amount of water added and the final volume being noted. The solution is saturated with CO and reduced by the addition of a little solid $Na_2S_2O_4$. Then CO is bubbled through again and after a few minutes enough saturated ammonium sulfate is added to about half saturate the solution. Crystals usually appear within 10 minutes. They have never failed to appear within 18 hours. In dilute solutions of hemoglobin, crystallization is slow and sensitive to the pH and ammonium sulfate concentration. In concentrated solutions such as are readily obtained by dissolving the ammonium sulfate precipitate, it is difficult not to get crystals.

The preparation from cyan methemoglobin (normal or "reversed") of HbCO crystals free of cyan methemoglobin, ammonium sulfate, and the reducer $Na_2S_2O_4$ (Stokes' reagent does not reduce cyan methemoglobin) is a tedious matter requiring a lengthy series of dialyses. First, the ammonium sulfate precipitate is dissolved by dialysis against water. This solution is converted into HbCO, dialyzed

against an alkaline solution to remove the $Na_2S_2O_4$ and ammonium sulphate, then dialyzed against an isoelectric solution of pH 6.8 and finally concentrated and crystallized by pressure dialysis. All the dialyses are done at 5°C. in a shaking dialyzer (Kunitz and Simms (3)) with collodion membranes. $Na_2S_2O_4$ even in small amounts makes collodion membranes fragile, so solutions should not be left in the membranes longer than a day, preferably less. In any case, collodion membranes are soon made impermeable by contact with hemoglobin solutions.

The procedure in more detail is as follows:

First, in order to dissolve the ammonium sulfate precipitate without too much dilution it is dialyzed for an hour against distilled water. The solution is then put in an Erlenmeyer flask which is evacuated and filled with CO. One-fifth of a volume of a 1 per cent solution of $Na_2S_2O_4$ in 0.5 per cent NH_3 is then added and the flask evacuated and refilled with CO. The solution is allowed to stand 10 minutes and is then shaken and dialyzed at least overnight against a 1/20 M solution of K_2HPO_4 or any similar slightly alkaline buffer solution. It is then further dialyzed overnight against water or an isoelectric phosphate buffer and finally pressure dialyzed and put away in the cold to allow time for crystallization. The dialysis solutions all are saturated with coal gas and the membranes all contain bubbles of pure CO. The HbCO may be crystallized out of any desired solution in which it is sufficiently insoluble and may, of course, be recrystallized.

SUMMARY

The coagulation of hemoglobin is probably reversible.

Several methods are described for preparing soluble crystalline hemoglobin from hemoglobin denatured by HCl or trichloracetic acid.

The yield is about 75 per cent.

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THE CATALYTIC EFFECT OF DYES ON THE OXYGEN CONSUMPTION OF LIVING CELLS

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It has been shown (1) that methylene blue added to living cells increases their oxygen consumption, the increase being partly due to the oxidation of some of the decomposition products of the carbohydrate metabolism (2). It was then suggested (3) that the action of methylene blue was due to its reversibility with respect to oxidation, which enabled it to play the rôle of catalyst in the presence of atmospheric oxygen. It was of interest to study the relation between the reduction potential of the dye and its catalytic power and to correlate these properties with the reduction intensity in the cell.

The most suitable material for this purpose is the mature unfertilized eggs of starfish (*Asterias forbesii*).

Their pH value and reduction intensity have been studied and their active respiration allows accurate measurements of oxygen consumption; furthermore, the more or less transparent protoplasm of the eggs makes it easy to observe the penetration of the dye through the cell wall. The eggs of the same starfish were used in each series of experiments. They were collected in flat-bottomed dishes, washed once with sea water, and after 40 to 50 minutes (time necessary for maturation) the experiments were started. The oxygen consumption was measured at 25°C. ±0.01 in Warburg's vessels with Barcroft micromanometers. Freshly prepared dyes were used at a concentration of 1.253 millimols per liter. The oxygen consumption of the cells was measured for an hour, after which time, the dye kept in the side arm of the respiration vessel was thrown into the main vessel containing the cells, and the oxygen consumption again measured for 1 hour.

Relation Between the Reduction Potential of Dyes and the Increase in the Oxygen Consumption of Living Cells

We possess now a fair number of reversible dyes which can be employed as indicators of reduction intensity. We selected them,

having in consideration these two factors: (1) permeability of the cell membrane to dye and (2) the toxicity of the dye to the cell. We, therefore, had to discard those reversible dyes which either did not penetrate the cell membrane or were clearly toxic. Cohen, Chambers and Reznikoff (4), in their studies on the reduction potentials of *Amoeba dubia*, studied the toxicity of some of these dyes after microinjection. The following dyes which cover a wide range of reduction intensity were employed: (1) Phenolindophenol; (2) toluylene blue chloride;* (3) cresyl blue† (4) methylene blue† (5) cresyl violet; (6) safranine; (7) Janus green; and (8) neutral red.

TABLE I

The Effect of Reversible Dyes on the Oxygen Consumption of Starfish Eggs (Mature, Unfertilized). Dyes Which Penetrate through the Cell Membrane

Name of dye	Oxygen consumption per hour in c.mm.		Number of experiments	Per cent increase
	Before dye addition	After dye addition		
Phenolindophenol	19.2	39.9	12	104
Toluylene blue chloride	32.0	121.6	9	280
Cresyl blue	22.3	82.5	11	270
Methylene blue	23.5	86.7	18	269
Cresyl violet	25.2	66.6	15	165
Safranine	24.3	30.7	14	26
Janus green	23.8	26.2	13	10
Neutral red	25.0	24.5	14	None

A number of experiments were performed with each dye and the results tabulated in Table I represent the averages of the figures obtained. There is always a difference in the catalytic power of the same dye upon different samples of eggs, one cause being the number of cells employed, which alter the concentration of the dye in relation to the individual cells. This difference, however, becomes small when the same quantity of eggs is used. The relation between the E'_0

* We are indebted to Dr. Barnet Cohen for this sample of toluylene blue chloride.

† No claim is made for the purity of the methylene blue from azure and it may be open to further investigation whether the effect of methylene blue is in part to be ascribed to the content of azure.

(the electrode potential difference between the normal hydrogen electrode and an equimolecular mixture of oxidant and reductant at pH 7.00) of the dye at the cellular pH (pH of the protoplasm of mature starfish eggs, 6.8±0.1) and its power to act as a catalyst for cellular oxidations, can be seen in Table II where the E'_0 of the dyes has been tabulated with the corresponding increment produced in the cell oxygen consumption. The statement made previously (1) that

TABLE II

The Effect of Reversible Dyes on the Oxygen Consumption of Mature, Unfertilized Starfish Eggs. Relation between Their Catalytic Power and E'_0

Name of dye	E'_0 at pH 7.00 in volts	Measured by	Increase in the O_2 consumption per cent per hour
Phenolindophenol	+0.229	Cohen, Gibbs and Clark (5)	104
Toluylene blue chloride	+0.115	Phillips, Clark and Cohen (11)	280
Cresyl blue	+0.035	Rapkine, Struyk and Wurmser (12)	270
Methylene blue	+0.011	Clark, Cohen and Gibbs (5)	269
Gallocyanine	+0.013	Measured in Professor Michaelis' Laboratory (not yet published)	46
Indigodisulphonate	−0.125	Sullivan, Cohen and Clark (5)	None
Cresyl violet	−0.165	Rapkine, Struyk and Wurmser (12)	165
Safranine*	−0.255	Vellinger (13)	26
Janus green*	−0.257	Rapkine, Struyk and Wurmser (12)	10
Neutral red*	−0.315	Vellinger (13)	None

* The values for the E'_0 of safranin, Janus green and neutral red, seem subject to caution. We have titrated commercial preparations of Janus green and neutral red and found that both of them were partially irreversibly oxidized; therefore no definite value for the E'_0 could be found. A single experiment with safranin gave identical results. Professor Clark informs us he has had the same results with these dyes.

reversible dyes with an E'_0 close to that of methylene blue are the best catalysts, holds true.

As a result of the fundamental researches of Clark and his coworkers (5), who have laid the foundations upon which to build our knowledge of oxidation-reduction systems of biological interest, many attempts have been made to measure the reduction intensity of living cells.

The reduction potential of the eggs of starfish has been measured by the method of microinjection of reversible dyes by Needham and Needham (6), Rapkine and Wurmser (7) and recently by Chambers, Pollack and Cohen (8). While the first two investigators place the aerobic rH of starfish eggs at 19 to 20, Chambers and coworkers have found it to be approximately 12. We accept the last figure as the reduction potential of the eggs of starfish for they are of the same species we have been employing. As an rH of 12 corresponds to a potential of −0.060 volt when referred to the standard hydrogen electrode, it can be seen that the dyes with optimum catalytic power are those whose E'_0 lies on the positive side of the reduction potential of the cell we are studying. When the E'_0 of the dye is much more positive than the reduction intensity of the cell, the effect of the dye will depend on its concentration. This easily can be observed, using phenolindophenol as catalyst. Accidentally the concentration we employed was just enough to overcome the reduction capacity of the cell and phenolindophenol then acted as a catalyst. But upon diminishing the concentration from 1.253 mM to 0.79 mM, the dye was completely reduced by the starfish eggs, and kept reduced. No catalytic effect was then observed and the oxygen consumption remained at the same rate as before the addition of the dye. The same eggs with the usual concentration of phenolindophenol (1.253 mM) gave an increase of 106 per cent. When the E'_0 of the dye lies on the negative side of the reduction potential of the cell, the catalytic power becomes lower, until with dyes whose E'_0 is −0.200 volt more negative than the cell reduction potential the catalytic effect ceases.

The Effect of the Permeability of the Cell Membrane

The experiments of Wieland and Bertho (9) on the fermentation of alcohol to acetic acid by *Bacterium orleanenses* in the absence of free oxygen due to the dehydrogenating action of dyes, have shown that while benzoquinones, which readily penetrate the cell walls, produce a rapid dehydrogenation, methylene blue, which does not penetrate, acts slowly, as in this case the dehydrogenation takes place only on the outer surface. The same phenomenon can be observed in the catalytic action of dyes on the oxidative processes of starfish eggs in an atmos-

phere of oxygen. Taking into consideration the relation of the E'_0 of the dye to its catalytic power, we arranged the experiments in the following manner: (1) A dye which does not penetrate the cell membrane and with an E'_0 more positive than the reduction potential of the starfish eggs, was compared with a dye with similar E'_0 but which does penetrate. The pairs of experiments were always performed with the same sample of eggs. The effect of gallocyanine was accordingly compared with that of methylene blue (Table III). While the latter increased the oxygen consumption by 260 per cent in these experi-

TABLE III

The Effect of Reversible Dyes on the Oxygen Consumption of Starfish Eggs (Mature, Unfertilized). The Effect of the Membrane Permeability

Name of dye	E'_0 at pH 7.00 in volts	Oxygen consumption per hour in c.mm.		Permeability of membrane	Per cent increase
		Before dye addition	After dye addition		
Gallocyanine*	+0.013	23.1	33.7	Does not penetrate	46
Methylene blue	+0.011	23.3	85.5	Penetrates	267
Indigodisulphonate	−0.125	25.4	24.6	Does not penetrate	None
Cresyl violet	−0.165	25.3	65.8	Penetrates	160

* A detailed investigation on the properties of this reversible dye will be published by Dr. L. Michaelis.

ments, the increase produced by gallocyanine was only 46 per cent. As gallocyanine does not penetrate through the cell surface of the starfish egg, this observed increase must be ascribed to the action of the dye on the oxidative processes taking place at the outer surface of the cell. (2) In a similar manner, experiments were performed with dyes possessing an E'_0 more negative than the reduction potential of starfish eggs, namely, indigodisulphonate and the slightly more negative cresyl violet (Table III). In this latter case, even those oxidative processes taking place at the outer surface of the cell were not activated by the dyes, their effect being negative.

DISCUSSION

1. Relation Between the Catalytic Power of the Dyes and Cellular Metabolism

The ability of reversible dyes to catalyze certain oxidative processes taking place in the living cell seem to us of importance for the understanding of the mechanism of some phases of cellular metabolism.

There are within the cell two kinds of oxidative processes: (1) The respiratory process which we can define with Warburg (10) as the combustion of organic substances through molecular oxygen in the living cells, a process catalyzed by an iron-containing enzyme, which, so far as Warburg's researches show, is the same for a wide variety of cells and probably for all cells. (2) The oxidative processes which require the activation of the organic substances and the presence of a hydrogen acceptor. The former, *i.e.*, the respiratory process, is inhibited by cyanide and CO and is activated by a ferment present in the same form in every cell; the latter is not inhibited by cyanide and is effected by multiple hydrogen acceptors. The long still existing controversy between Warburg's conception of respiration and Wieland's theory of oxidative dehydrogenation has been kept alive because of the different and distinct definitions given to the term respiration. The oxidative processes activated by reversible dyes belong to the second category of oxidations. They have in common with the respiratory process, the consumption of oxygen, the production of carbon dioxide, and the inhibiting effect of strongly adsorbable substances (narcotics), but they differ from respiration in that they are intimately related to the fermentative process. The effect of the dye appears to be proportional to the fermentative power, *i.e.*, the anaerobic glycolysis of the cell, as we will show in a later communication.

The relation of the E'_0 of the dye and its catalytic power to the reduction intensity of the cell is suggestive. Those dyes possessing an E'_0 on the positive side of the reduction potential of the cell are the more active, their activity being diminished or even abolished only when the dye is far beyond the reduction intensity or when the amount of dye used has been lower than the reduction capacity of the cell. The dyes whose E'_0 is on the negative side of the reduction potential of the cell have less effect, and, beyond certain limits, none. If we

plot on the abscissa the E'_0 of the dyes at pH 7.00 (pH of the protoplasm) and on the ordinate the per cent increase of the oxygen consumption of the cell due to the addition of the dye (Fig. 1), we have a plateau-like curve extending from the reduction potential of the starfish eggs, equivalent to −0.06 volt (referred to the standard hydrogen electrode), to +0.115 volt. On either side of this plateau the curve

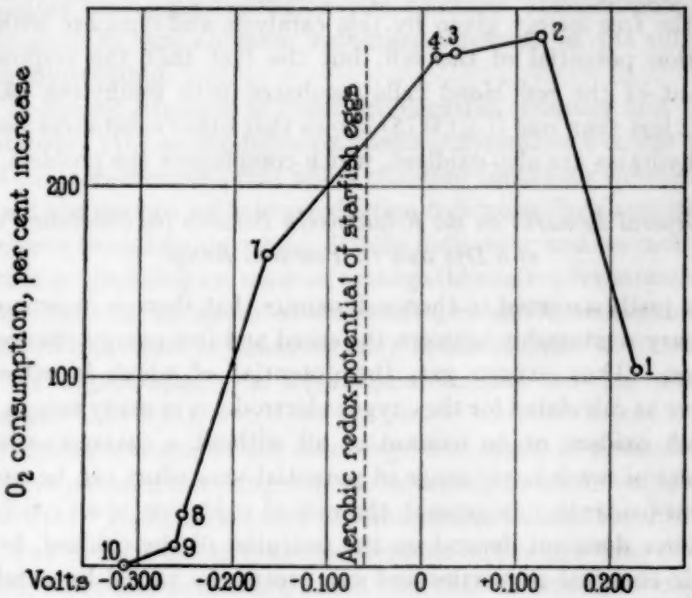

FIG. 1. Relation between the catalytic power of dyes and its E'_0 to the reduction potential (aerobic) of starfish eggs. (Dyes which penetrate through the cell wall.) Abscissa = E'_0 of dyes at pH 7.00. Ordinate = per cent increase of O_2 consumption after dye addition. 1, Phenolindophenol. 2, Toluylene blue chloride. 3, Methylene blue. 4, Cresyl blue. 7, Cresyl violet. 8, Safranine. 9, Janus green. 10, Neutral red.

falls abruptly. It seems possible that the substances responsible for the reduction potential of the cell are those activated by the dyes as catalysts. If the aim of those investigators studying the reduction potentials of cells is to correlate them with certain phases of the cellular metabolism it is indeed natural that we should look for a correlation between the appearance of certain metabolic products and the attain-

ment of certain levels of reduction intensity. It has been shown by one of us and Harrop (2) that one of the substances oxidized by the catalytic effect of reversible dyes is a carbohydrate, as the amount of lactic acid produced by the blood cells when incubated in the presence of methylene blue is less than the normal control. If the carbohydrates were the only substances oxidized by the action of dyes, it would be possible, from the entropies of sugar and lactic acid, to determine the free energy given by this catalysis and compare with the reduction potential of the cell; but the fact that the respiratory quotient of the red blood cells incubated with methylene blue is always less than one ((±) 0.75) shows that other substances besides carbohydrates are also oxidized, which complicates the problem.

II. General Remarks on the Relationship Between the Catalytic Power of a Dye and Its Potential Range

It is justly assumed in thermodynamics that there is in general no necessary relationship between the speed and free energy change of a reaction. Thus oxygen gas, the potential of which is extremely positive as calculated for the oxygen electrode, is in many cases a very sluggish oxidant or no oxidant at all without a catalyst; whereas oxidants of much lower range of potential very often can be used as efficient oxidants. In general, the rate of oxidation of an oxidizable substance does not depend on the potential of the oxidant, but on specific chemical properties and can greatly be varied by catalysts. The action of catalysis, indeed, is a much more important factor for the rate of oxidation than the potential range of the oxidant. From this standpoint it seems difficult to understand how the speed of oxygen consumption in living cells by the action of dyes could be referred to the potential of the dye. This contradiction may find an explanation in the following way. Though, in general, speed and potential have no relationship, yet there are quite definite cases in which such relationship can be recognized.

1. We have first the case described by Conant (14) when he reduces a substance capable of an irreversible reduction (a number of azo-dyes, nitrocompounds and unsaturated 1,4,diketones) by an equimolecular mixture of the reduced and oxidized forms of a reversible reductant. No equilibrium between these two substances can be established.

What happens is that the substance in question will be reduced first with a certain velocity, then this velocity will gradually slow down and finally become so slow that we may speak practically of a quasi equilibrium. This is not a thermodynamic state of equilibrium, but it is a state in which the reaction velocity is almost zero. Conant has found that the reduction in an homogenous solution of such compounds is a function of the potential of the reducing agent. He designates that potential which just produces a measurable speed of reaction, as "the apparent reduction potential" of the substance being investigated.

2. A second example is found in Voegtlin, Johnson and Dyer's experiments (16) on the reducing power of normal and cancer tissue. These authors observed that under standard conditions the reduction time of the various oxidation-reduction indicators decreases with an increase in the electrode potential of the indicators; and the indicators, if tested by the biological method, arrange themselves in the same order as that obtained by means of the purely physical electrode measurements. From their experiments they conclude that "the reduction time is approximately a logarithmic function of the electrode potential."

3. A third example is the following: The speed of oxidation of a leuco-dye by molecular oxygen seems to depend on the potential range of the dye. There are scarcely accurate data available for this assertion, and the velocity of oxidation depends in this case also on other factors. For instance, Clark found (15) that the speed of oxidation of methylene white by oxygen is approximately proportional to the fifth root of the hydroxyl ion concentration, but, though other accurate data are not known, there is one striking fact which can be used in this connection. The leuco-dyes in a solution of approximately the same pH are oxidized by molecular oxygen at a very different rate, which quite obviously depends on the potential range of the dye. Reduced indophenol is rather slowly oxidized in the air, and in a gas mixture of very low oxygen pressure is even re-oxidized very sluggishly. Methylene white is oxidized at reduced oxygen pressure quicker than indophenol indeed, but only gradually too. Reduced safranine is reduced even at low oxygen pressures with great speed. It is very sensitive indeed for traces of oxygen, so it seems likely that the speed

of oxidation by molecular oxygen is related to the potential range of the dye.

In these considerations there is involved an explanation for the different catalytic effects of the various dyes on cellular oxidations. When a dye is added to a suspension of cells, the dye will be reduced by the cell with a definite velocity, and the reduced dye will be oxidized by the air with a different velocity, also depending on the conditions. The catalytic power of the dye depends on these two velocities. If the velocity of the oxygen consumption brought about by the dye as a catalyst is greater than the one produced by the natural catalyst of the cell, then the dye will increase the oxygen consumption. In the case of indophenol the speed at which the dye is reduced by the cell is very great; but the speed at which the reduced indophenol is reoxidized by air is very slow, so slow indeed that indophenol does not increase the oxygen consumption unless in high concentrations. In the case of methylene blue the dye is easily reduced by the cell, and the reduced dye is easily oxidized by the air, a fact which makes methylene blue a good catalyst for cellular oxidations. On the other hand the reduction of safranin by the cell is extremely slow though its reoxidation by air is very rapid. Therefore, the dye will not act as catalyst. This seems a satisfactory explanation for the fact that the potential range of a reversible dye has a recognizable relationship to its ability to work as an oxidation enzyme.

SUMMARY

From the experiments described in this paper and in those previously published it can be concluded that dyes which can be reversibly oxidized and reduced, act as catalysts for some oxidative processes taking place in the living cells, as is manifested by an increase in their oxygen consumption.

It has been found that the catalytic power of the dyes on the oxygen consumption of starfish eggs (mature, unfertilized) is conditioned by two factors: the reduction potential of the dye and the permeability of the cell surface. Dyes whose E'_0 is towards the positive side of the aerobic reduction potential of the starfish eggs have a maximum catalytic effect. This catalytic power decreases as the E'_0 becomes more negative than the reduction potential of the cell and becomes

nil beyond certain limits. When a dye cannot penetrate into the cell, its effect is greatly diminished as in this case only those oxidative processes taking place at the outer surface of the cell can be activated.

Whether a dye can act as a catalyst or not is dependent on whether the normal consumption of oxygen by the cell is slower or quicker than the oxidation activated by the dye. The speed of this activation is correlated to (1) the speed at which the dye is reduced by the cell, and (2) the speed at which the leuco-dye is oxidized by the atmospheric oxygen. If one of these two processes is slower than the normal respiration, the dye cannot increase the rate of oxygen consumption (phenol indophenol at low concentrations which is kept reduced by the cell is very slowly reoxidized by atmospheric oxygen, on the other hand safranin and neutral red which are not reduced by the cell or at least too slowly reduced, though rapidly reoxidized by air). It will depend on these two reactions velocities whether a dye will act as catalyst (methylene blue and dyes with similar E'_0 which are quickly reduced by the cell and the leuco-dyes of which are relatively quickly reoxidized). Though this relationship between the reduction potential of the dyes and its catalytic power would seem in contradiction with the well known thermodynamic assumption that there is in general no distinct relationship between the potential and velocity of the reaction, we have pointed out from the literature some of the various experiments where one does recognize this connection.

Our sincere thanks are due to Prof. L. Michaelis, for his kind help and for much valuable advice and suggestions.

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THE DIRECT CURRENT RESISTANCE OF NITELLA

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PLATES 6 AND 7

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Organisms in which the effective direct current resistance of intact protoplasm may be measured are necessarily few, since in most tissues the resistances of intercellular spaces, walls, vessels, etc., remain uncertain. In suspensions of cells the volume is difficult to estimate accurately; in closely packed cell masses centrifuged therefrom, some liquid still remains between the cells, and certainly many cells are injured or killed by the treatment (as may be seen if one attempts to re-suspend the mass).

The injury probably accounts for the low specific resistance of such masses. The figures given by McClendon[1] illustrate this: his values for specific resistance (low frequency A.C., and D.C.) range from 324 ohms for ox erythrocytes in serum to 3944 ohms for hog erythrocytes in sugar solution. Since such a cubic centimeter volume is several thousand cells thick, with twice that number of cell surfaces placed in series and presumably additive in resistance, it is seen that the maximum individual surface resistance is *1 ohm or less per square centimeter*. McClendon[2] gives the value of 0.1 ohm for beef erythrocytes. This very low figure is probably due to leaks and to injured cells although it is possible that erythrocytes are exceptional in their low resistance.

In the large single cells of *Valonia* the resistance of the shunt around the cell (cellulose wall) may be much more satisfactorily estimated by actual isolation, *i.e.*, replacement of the protoplasm and sap by air.[3] Difficulties of maintaining the wall (imbibed with sea water) in the same condition as in life prevent the measurements from being absolute, but the calculated value of 10,000 ohms or more per square

[1] McClendon, J. F., *Protoplasma*, 1929, **7**, 561.
[2] McClendon, J. F., *J. Biol. Chem.*, 1926, **69**, 733.
[3] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

centimeter of protoplasmic surface, in the majority of cells, is much higher than has been demonstrated previously by direct methods.

In general the higher the value of the shunting resistances (walls) the more accurate will be the measure of those in the protoplasm. For this purpose a fresh water organism, with walls imbibed with tap water or distilled water, is more advantageous than a marine one, necessarily covered with well-conducting sea water. The long multinucleate cells of the fresh water plant *Nitella* (*N. flexilis* has been chiefly employed) meet these conditions very well and are shown in the present paper to have a high effective protoplasmic resistance.

Method

The chief technical difficulty is the measurement of resistance under the application of very small potentials, in order to avoid stimulation[4] and injury, with their persisting effects on normal resistance values. This is accomplished by the direct current bridge with vacuum-tube detector, previously described.[3] With this circuit a galvanometer sensitivity of 1 mm. for a 1 per cent unbalance of the bridge is obtained when only 50 mv. are applied to resistances as high as 3 or 4 megohms; a balance can be made to 5 per cent under applications of 10 to 15 mv. The current flow in the latter case is less than 0.005 microampere.

The usefulness of this bridge depends on the detector being actuated by differences of potential only, and not by the absolute current flow through any arm. When a given potential is applied to the bridge, the detector responds with a constant deflection for a given ratio of resistances, whether the actual resistances are large or small. It is thus available for very high resistances, and can be used to follow changing resistances as a deflection instrument without balancing.

In order to use the equal ratio arm bridge at these high resistances, an extra decade of 100,000 ohm steps was constructed of selected wire-wound "Electrad" resistors, with baked enamel coverings. These were accurate to 1 per cent and had a very small phase angle, giving no perceptible reactance when balanced against electrolyte or metal film resistances. For standards and ratio arms of 1 megohm, frequently calibrated "grid leaks" (of metal film on glass), were used. For the lower values (1–100,000 ohms) and the 1000 ohm ratio arms, General Radio Company resistances were used.

The general circuit and the use of the vacuum tube detector are described in

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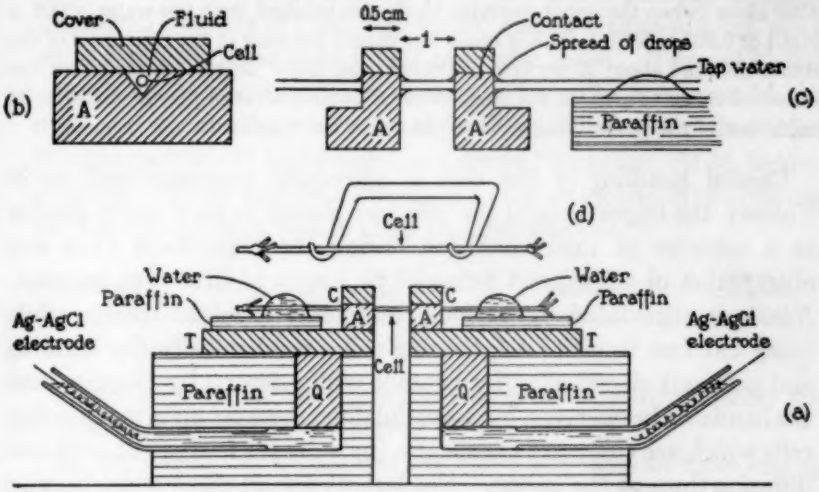
[4] Blinks, L. R., Harris, E. S., Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 836.

a previous paper.[3] In order to avoid stimulation by potential differences existing between the contact points on the cell, these P.D.'s were balanced out by an equal and opposite potential from a potentiometer in series. The existence of such potentials almost always signifies that stimulation has occurred shortly before. With carefully treated cells they are absent or very low.

The apparatus is shown in Text-fig. 1. As with *Valonia*, the cell wall is made the only electrical leak around the protoplasm. The cell is supported by agar blocks in order to control the dimensions and the area of current flow. For uniformity



TEXT-FIG. 1. Apparatus used for measuring resistances of *Nitella*. *a*, electrodes and holders with cell in place. The outer ends of the cell rest on paraffin blocks to insulate them from the stage *T*. A pool of tap water on these blocks keeps the cells from drying out. *b*, end view of agar block *A* with cell in the slot, covered with agar to make contact all round. The slot is filled with the desired solution. *c*, side view of blocks (*AA*) showing arrangement for a 0.5 cm. length of contact. *d*, glass cradle for lifting cells out of water and upon contacts.

these blocks are cut by a spaced pair of blades to be 1 x 1 cm. in cross section, and are about 3 cm. long. These are imbibed with the desired solution. They can be combined in groups of 2 or 3 to give longer contacts. For shorter contacts part of the block is cut away as shown in the figure, giving for example 0.5 cm. Slots are cut down about 2 mm. into the surface to receive the cell and to keep it covered with a small amount of fluid. To insure more certain contact all around the cell a smaller agar cover may be placed over the slot. The air gap (*l*) is measured, or spaced by one of the cut blocks which is then removed. It may be varied by sliding the blocks upon intermediate agar stages, *T*. The latter are usually imbibed with tap water or dilute NaCl, and rest in turn upon the plugs *Q*, which are

set into the paraffin blocks rather tightly and are imbibed with 0.1 M NaCl. They make contact with the electrodes through more 0.1 M NaCl, which is renewed from time to time to equalize the potentials of the electrodes.

The electrodes may be quite small, due to the very small current densities sent through them; coiled silver wire, frequently recoated with chloride, is very satisfactory, and the photographic records show such electrodes to have only very slight polarization with much larger current densities than are used in the experiments. The resistance of the conducting system up to the cell is from 3000 to 8000 ohms (when the upper movable blocks are imbibed with tap water, 0.001 M NaCl or 0.001 M KCl). This is much less than 1 per cent of the resistance of the intact cells and about 10 per cent of that of dead cells. It is subtracted from the observed values to give the net resistances (R), and allowance may be made for its reduction of the potential applied to the cell, when significant.

Careful handling of the cells is extremely necessary and, as in *Valonia*, the importance of the *previous* history is very much greater as a criterion of their condition during the experiment than any observation of subsequent behavior or length of life. For instance, *Nitella* is stimulated by various treatments it might receive while being cut free from its neighboring cells: mechanically (by bending and pulling); electrically (by contact with scissors, by charges from the hands of the operator, by potential differences set up in neighboring cells which are cut); and chemically (by salts set free at cut ends and diffusing through the water). The latter leave an effect which may be detected for a day or more by markedly lower resistances. The effects of KCl are especially slow to disappear.

Since some of these stimulations are impossible to avoid in the preparation of the cells, it is necessary here, as with *Valonia*,[3] to reach uniformity by keeping the separated cells for some time under favorable conditions. Two days sojourn in tap water or distilled water is sufficient to bring most of the *Nitella* cells to a rather high resistance, but the longer the recovery the better their condition. Single cells from cultures kept 2 or 3 months in covered pans in diffuse light reach much higher resistances, as will be seen from the data to be presented.

All these precautions are of little avail if in placing the cell in the apparatus it is subjected to bending or is handled by metal forceps or by the fingers conveying static charges to it. If a cell is observed immediately after such treatment, it will almost invariably display large potentials representing more or less of its recovery from stimula-

tion. The cell must be handled with glass or bone-tipped forceps and preferably lifted out of water supported from below on a glass cradle (Text-fig. 1 *d*) in which it is lowered upon the agar contacts.

Resistance of Cells

It is instructive to consider the variation in cells freshly cut from the plants and later recovering from the effects referred to above. (Standard contacts of 1 cm. (tap water) and an air gap of 1 cm. are to be understood throughout, unless otherwise noted. Temp. 17°–20°.)

A group of plants was cut 1 week after collection, and 53 cells measured within 1 hour after cutting. The cells were distributed into two groups: (*a*) 25 had a resistance above 1 megohm; (*b*) 28, below 1 megohm. These were kept overnight in tap water and in the morning the two groups were nearly alike (one cell had died):

Group	Below 1 megohm	1–1.5 megohms	Above 1.5 megohms
(*a*).....	8	10	7
(*b*).....	9	8	10

On the third day all but 2 cells had a megohm or more resistance and 17 cells had reached 2 megohms or higher.

On the fourth day 4 cells had reached 3 megohms or more and an equal number were above 2 megohms. All the rest were distributed between 1 and 2 megohms. In general the latter values hold for most cells which are used for daily experimentation.[5]

Very careful handling and segregation of *Nitella*, however, causes its average resistance to go still higher. This was the case with a series of cells which had been in the laboratory over 2 months, undisturbed in a pan of water covered with a glass plate. In a group of 26 such cells, 8 had 3 megohms resistance or higher, and only 5 had less than 2 megohms—3 of these after accidental stimulation. Thus half the cells lay between 2 and 3 megohms. Two days later much the

[5] In some groups of cells the resistance may remain markedly lower for a much longer time than is here indicated. The conditions of this reduction are not fully understood but probably involve the composition of the New York City tap water, which at times is definitely toxic. The effect seems to be a much longer retention of salt in the protoplasm, giving resistances of 0.3 to 0.5 megohm. These cells are, however, capable of stimulation and give good action potentials.

same values held, the only cells (3 in number) below 2 megohms being those which had been stimulated accidentally in measurement.

With these values for reference it is of interest to compare the resistances of living cells with those of dead cells. Killing with chloroform or high potentials (1 volt, frequently reversed), brings the value very uniformly to 50,000 or 60,000 ohms. This is true for all cells of the average diameter (0.05 cm.).

If a dead cell is allowed to stand in tap water the salts diffuse out and the resistance rises in time to a high value again, often to about 2 megohms. A dead cell kept several hours in distilled water may have 5 megohms or more resistance.

These values are in the range given for healthy cells, but there is no difficulty in distinguishing between such water-filled walls and the living cells. Aside from visible criteria such as color, turgor, regularity of chloroplasts, and cyclosis, there are electrical differences. Thus:

1. Living cells show characteristic "transient" effects at the application and removal of even the lowest potential (Plates 6 and 7).

In the study of these quick responses the string galvanometer (connected through the vacuum tube detector) is of great advantage over the ordinary galvanometer of the Wheatstone bridge. Photographic records of the projected string image show these transients as abrupt movements away from the zero line, when the battery circuit is closed and opened (the bridge having previously been balanced to the steady state).

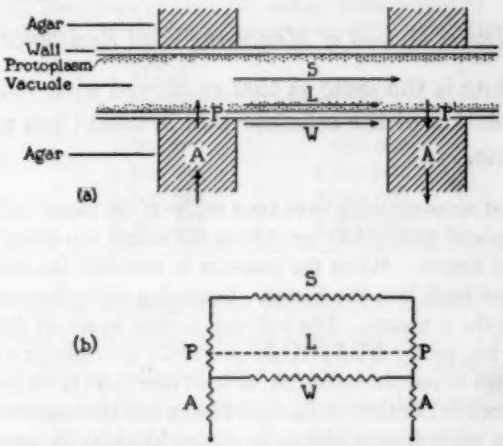
The downward movement in each record occurs with the closing of the battery circuit (make). Current begins to flow through the bridge, reaching a steady value nearly instantly in each arm except that containing the living cell. Through the latter a larger current at first starts to flow, producing a downward jump of the string, but a back E.M.F. builds up, which decreases the current flow until a smaller constant value is reached. The string returns in a regular curve to the zero line representing the balance for the steady state.

At "break" there there is an equivalent upward movement due to the back E.M.F. which has been developed. As this is discharged the image returns to zero, representing no current flow in the bridge.

These phenomena will be considered more fully in a later paper. For present purposes large transient effects may be regarded as evidence of normal condition (Plate-fig. 1 *a*). Injured cells show much shorter transients (Plate-fig. 1 *b*), and dead cells are practically non-reactive (Plate-fig. 1 *c*).

2. When the applied potential is increased to between 20 and 50 mv. the living cells almost always undergo stimulation, giving typical diphasic action potentials as high as 125 mv. (Plate-fig. 1 *d*). Dead cells show no such effect.

3. Increase of applied potential to 100 mv. or more causes a falling off of resistance in living cells, the more marked the higher the potentials, while dead ones have a constant resistance over a wide range of current density.



TEXT-FIG. 2. (a) Diagram of possible current paths in the *Nitella* cell; (b) equivalent circuit. *AA*, agar blocks. *PP*, across protoplasm; *L*, along protoplasm; *W*, along wall; *S*, through vacuole.

Resistance of Protoplasm

When comparison is made between a living cell and a dead one taken immediately after killing (before salts can diffuse out) it is seen that there is an immense reduction in resistance; in the case of good cells it may be from 3,500,000 ohms (live) to 60,000 ohms (dead). Thus the living value is over 50 times the dead value, which is comparable to the ratio observed in *Valonia*,[8] and is much greater than in most other organisms hitherto reported upon. It is evident therefore that the intact protoplasm must have at least the resistances indicated (from 1,000,000 to 3,500,000 ohms) and may have more, depending on the value of the leaks around the cell. (This, of course, depends on the salt content of the wall, which would increase by diffusion from

the sap on injury or death.) Text-fig. 2 shows the possible circuits in the cell. (R is the resistance of the cell as measured in the bridge.) If there were no leak along the wall ($W = \infty$), R would equal $2P$, and P would range from 0.5 to 1.75 megohms. For an area of 0.16 sq. cm. at each contact, this gives a value of from 83,000 to 290,000 ohms per square centimeter of protoplasmic surface. But the cell wall is a shunt of definite resistance, which may be estimated by the direct method.

Direct Method of Measuring Wall Resistance

The procedure is the same as that employed with *Valonia*[3] and involves the isolation of the cellulose wall in conditions comparable to the living state.

After sufficient measurements have been made on the intact cell, it is cut at one end and the sap and protoplasm are driven out under tap-water, by pulling the cell through the fingers. When the pressure is removed the wall expands again and water rushes back into the lumen. Squeezing the cell several times serves to wash out all the contents. The cell wall is then squeezed flat into a ribbon, removed to the air, gently dried and blown up with air through a capillary. The wall is stiff enough to remain expanded, at least over part of its length. This air-filled wall is placed in position on the agar blocks and thoroughly drenched on the outside with tap water from a pipette to restore its moisture content. Owing to the small diameter of the cell, excess drops do not tend to collect upon it in the air gap but move to the agar, where there is a slight spread of water by capillarity from the blocks out upon the cell (Text-fig. 1 *c*). This increases the effective area of contact, and must be allowed for.

It is found, if this experiment is carefully done and no liquid creeps along inside the lumen, that the resistance of the air-filled wall is from 5,000,000 to 10,000,000 ohms per centimeter of length, depending on its thickness, moisture content, and salt content of the water. If imbibed with distilled water the value is even higher.

Taking 6,000,000 ohms as a good value for the resistance of the wall (W), it is seen from the relation

$$S + 2P = \frac{W R}{W - R}$$

(as in Text-fig. 2) that P has the values shown in Table I, for cells of several observed resistance values (R). S may be neglected as being within the limits of measurement for most cases.

The area of contact (1 cm. long) for a cell of diameter 0.05 cm. is about 0.16 sq. cm. Thus for *Nitella* cells in good condition the effective resistance of protoplasm in contact with tap water ranges between 100,000 to 700,000 ohms per square centimeter of surface with the most common value from 200,000 to 250,000 ohms per square centimeter.

TABLE I

D. C. Resistance of Cells (R) and of Protoplasm (P)

R	$\frac{WR}{W-R}$	P	P/sq.cm.
ohms	*ohms*	*ohms*	*ohms*
1,000,000	1,200,000	600,000	100,000
2,000,000	3,000,000	1,500,000	250,000
3,000,000	6,000,000	3,000,000	500,000
3,500,000	8,400,000	4,200,000	700,000

The higher values represent the increase as the cells remain undisturbed in tap water, after separation from the plant. This rise seems to be produced by the diffusion of KCl out of the protoplasm into the surrounding water, or by its absorption into the vacuole. Thus the higher values of P probably are due to more nearly potassium-free protoplasm. (Replacing the cells in solutions of higher KCl content causes the decrease of resistance again, as is shown later.)

That this is the resistance *across* the protoplasm and not, for instance, that of a path along it parallel to the wall (L in Text-fig. 2), is shown by a relative method which consists in changing areas of contact, and length of gap between contacts.

Relative Measurement of Surface Resistance

If the current path through the living cell were chiefly along L, increasing the length of the gap should increase the measured resistance in direct proportion to the length. Table II shows that no such relation holds. Only a small increase occurs corresponding to the greater length of path through the sap, which is a much better conductor. The actual length of the gap therefore has only a small effect on the average values for *Nitella* cells.

On the other hand, the *area of contact* has a large effect, as is shown in Table II for a typical cell. Calculated in terms of P the resistances are as shown in Table III.

TABLE II

D. C. Resistances by the Relative Method

	Dimensions			Resistances			
	Diam. of cell	Length of contact	Length of gap (l)	Live cell (R)	Dead cell (S)	Cell wall filled with tap water	Cell wall filled with air (W)
	cm.	*cm.*	*cm.*	*ohms*	*ohms*	*ohms*	*ohms*
Nitella (gap varied)	0.05	1.0	0.5	2,000,000	40,000	1,000,000	3,000,000
		1.0	1.0	2,050,000	60,000	2,000,000	6,000,000
		1.0	2.0	2,120,000	130,000	4,000,000	10,000,000
		1.0	3.0	2,200,000	200,000	6,000,000	15,000,000
(contact varied)	0.05	0.5	1.0	2,400,000	70,000	2,100,000	6,000,000
		1.0	1.0	2,000,000	60,000	2,000,000	6,000,000
		2.0	1.0	1,300,000	60,000	2,000,000	6,000,000
		3.0	1.0	1,000,000	60,000	2,000,000	6,000,000
Chara coronata (diameter greater)	0.1	1.0	1.0	700,000	40,000	500,000	1,500,000

TABLE III

Relative Protoplasmic Resistances (Calculated from Table II)

	Length of contact	R	$\frac{WR}{W-R}$	P at contact	Area of contact	Surface resistance per square centimeter
	cm.	*ohms*	*ohms*	*ohms*	*sq. cm.*	*ohms*
Nitella	0.5	2,400,000	4,000,000	2,000,000	0.08	160,000
"	1.0	2,000,000	3,000,000	1,500,000	0.16	240,000
"	2.0	1,300,000	1,600,000	800,000	0.32	256,000
"	3.0	1,000,000	1,200,000	600,000	0.48	288,000
Chara	1.0	700,000	1,320,000	660,000	0.32	200,000

Length of gap = 1 cm. W = 6,000,000 ohms. (*Nitella.*)
W = 1,500,000 " (*Chara.*)

The resistances per unit surface are relatively constant and lie in the range calculated by the direct method. The value for 0.5 cm. contact is low because the effective contact is proportionally longer

than the measured one due to creeping of drops along the cell. Thus the effective contact is more nearly 0.75 cm. The larger contacts are less subject to this error.

Similar calculations based on varying the *length of gap* (Table II) give the following specific surface resistances:

Gap 0.5 cm.	500,000 ohms
1.0 "	250,000 "
2.0 "	210,000 "
3.0 "	200,000 "

Here again the most discrepant reading is that of the short length where there is the most uncertainty due to the creeping of fluid. An arithmetic average of all the values calculated from Table II is 268,000 ohms. Omitting the figures derived from 0.5 cm. contacts and 0.5 cm. gap the average value is 250,000 ohms per square cm. of surface.

Effect of Salts

As indicated in the *Valonia* paper[3] the significance of the high direct current resistance of living cells depends on our assumptions concerning the physical nature of the protoplasm. The data of the present paper may be taken further to rule out the surface as an inert, possibly lipoid film (acting like a dielectric and passing a few ions indiscriminately by leakage). Its function is more clearly indicated as an electrode, or phase reversible to some ions and not to others. The *Nitella* cell is very selective in its response to different salts.

The resistances recorded for tap water contacts are typical of those observed when the cells are in contact with more concentrated solutions of the chief salts of tap water. Thus the values are much the same with contacts consisting of solutions of NaCl or $CaCl_2$ even up to 0.01 M. Contacts with 0.01 M LiCl, NH_4Cl, and $MgSO_4$ give values not very different from these. 0.1 M NaCl at the contacts lowers the resistance somewhat but it may still be as high as 1,500,000 ohms with the standard contacts and is usually above 1 megohm.

Such high resistances may be due to (1) the low mobility of ions in the protoplasm; (2) the back E.M.F. developed by the flow of current. The latter is analogous to the polarization of an electrode in the presence of salts to which it is not reversible. While the mechanism of polarization at such a surface as that of protoplasm is not yet satis-

factorily explained, the obvious effect is that very little current flows after the transient effects are over. This may be taken as indicating that the effective permeability of the protoplasm for most of the common ions is low.

Solutions of KCl, on the contrary, have an exceptional effect in lowering the resistance. Even with contacts of 0.001 M KCl the cells seldom have resistances above 1.5 megohms and with contacts of more concentrated solutions they have proportionally less. Thus 0.01 M KCl at the contacts lowers the resistance to 0.4 or 0.5 megohm, and 0.1 M KCl to 0.1 megohm or slightly higher. Recovery from such exposures is very slow.

These reductions are parallel to those produced on the P.D. across the protoplasm by KCl, in distinction from NaCl, and it is quite probable that the reason is the same in both cases; namely, the greater mobility of the K^+ ion in the protoplasm. Unpublished calculations by Osterhout from concentration potentials show the mobilities of K^+ to be about 75.0 and Na^+ 2.2 when Cl^- is taken to be 1.0. It will be noticed that these are roughly parallel to the protoplasmic conductances. Thus in contact with 0.1 M KCl the protoplasm (minus sap) has a resistance of 50,000 ohms; in contact with 0.1 M NaCl it has a resistance of over 1,000,000 ohms. Assuming approximately equal concentrations of ions in the protoplasm the mobilities would give rise to these differences, qualitatively at least.

In contact with KCl solution, the polarization of the cell (as shown by the "transient" effects of Fig. 1) is also much lowered. This would likewise be expected if the protoplasm were an electrode reversible to K^+ but much less so to Na^+. It would polarize when Na^+ ions were presented but allow K^+ to pass rather freely. Further work on these effects is in progress.

Stimulation

It is an interesting property of the cells of *Nitella* to be stimulated by electric current, and to transmit that stimulus as a negative variation, giving a typical diphasic action current comparable to that observed in muscle and nerve.[4] The times of the rise and fall of the curve (Plate-fig. 1 *d*) and of the recovery period are long enough for measurements of resistance changes to be made at various parts of

the process. This is facilitated by photographic recording, using the bridge as a deflection instrument in the manner previously described,[3] so that many more points may be observed than if null balancing were attempted.

The study of these phenomena is not complete, but the outstanding effects on resistance may be indicated. The crest of the negative variation is really a depression of the P.D. at the contact nearly to zero. At the same time the resistance across the protoplasm is likewise greatly lowered and may fall momentarily to 0.1 megohm, about as in contact with 0.1 M KCl. This suggests that the cathodic stimulation may consist in the movement of sufficient K^+ ions in an outward current (from the sap to the external solution) to reach approximately such a concentration just outside the protoplasm. In the process of recovery it would be carried away by diffusion, while the P.D. and the resistance return to a higher value.

But, as noted earlier, even a single stimulation leaves the cell in a state of much lowered resistance for some time afterward. Continued stimulation causes the resistance to fall lower and lower until a level of about 500,000 ohms is reached (as if in contact with 0.01 M KCl.) Along with this fall of resistance, there is frequently a reduction of P.D. across the protoplasm, which affects the magnitude of the negative variation, even to its complete suppression. Polarization responses (transients) also become much smaller. There is a slow recovery from these effects if the cells are replaced in tap water, much like the recovery from exposure to KCl solutions.

It is possible that all these evidences of fatigue are simply due to an increase of KCl on the surface of the cell, but there may be in addition a further alteration of the protoplasm. This is suggested by the fact that recovery often takes place even under the continued flow of the same current which was sufficient to stimulate, and which presumably continues to carry K^+ ions outward at the same rate as before. That P.D.'s and resistances can largely return to normal during this flow obviously requires some further mechanism of restoration. More experiments are being carried out on the process of breakdown and recovery during constant as well as changing flows of current.

SUMMARY

The electrical resistance of *Nitella* cells to direct current is determined in a Wheatstone bridge, using a vacuum-tube detector, and string galvanometer. Very small currents are passed through the cells, to avoid stimulation. The galvanometer record shows typical transient effects in the living cells at opening and closing of the circuit, due to the development of back E.M.F.

With 1 cm. contacts of tap water, and 1 cm. between contacts the resistances of living cells are usually between 1,000,000 and 2,000,000 ohms. They go as high as 3,500,000 ohms when the cells are in the best condition. The resistance falls to about 50,000 ohms immediately after killing.

Leakage around the cell is small because the wall is imbibed with tap water. By measuring the resistance of the isolated wall (air-filled), and by varying the areas of contact with intact cells, the effective protoplasmic resistance is calculated. This varies from 100,000 to 700,000 ohms per square centimeter of surface, with a typical value of about 250,000 ohms per square centimeter.

This high resistance represents a low permeability for most ions, since the values are nearly as high with contacts of 0.01 M NaCl, $CaCl_2$, LiCl, NH_4Cl, and $MgSO_4$. The resistances are greatly reduced however by solutions of KCl, which is correlated with a high mobility of the K^+ ion in the protoplasm.

Electrical stimulation causes a marked reduction of resistance, which may be due to exomosis of KCl.

EXPLANATION OF PLATES 6 AND 7

FIG. 1. String galvanometer records of bridge balance using *Nitella* cells.

a) Normal cell. Three successive applications and removals of 15 mv. potential; *M*, make, *B*, break. Bridge balanced to 3 megohms. Detector sensitivity 3 divisions per mv. Time marks, 1 second.

b) Injured cell. Three successive applications and removals of 15 mv. potential. Bridge balanced to 500,000 ohms. Sensitivity as in *a*. Time marks, 1 second.

c) Dead cell. (I) Two applications and removals of 15 mv. potential. Bridge balanced at 1 megohm. (II) Same with bridge balanced at 60,000 ohms. Time marks, 1 second. Sensitivity as in *a*.

d) Normal cell, stimulated by a 50 mv. potential. A diphasic action current ensues. Sensitivity reduced; time marks, 5 seconds.

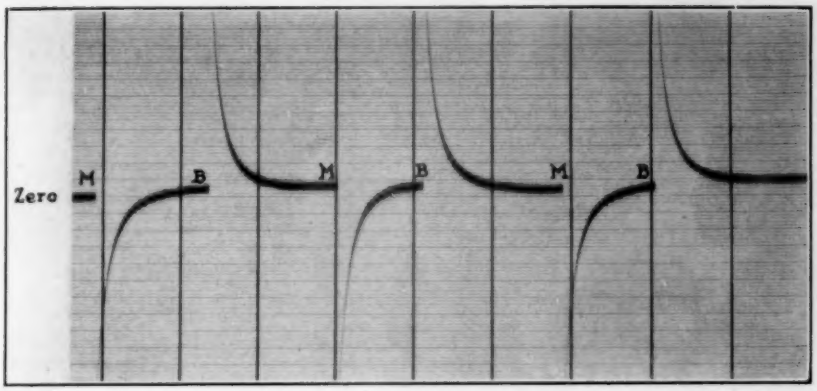


FIG. 1*a*

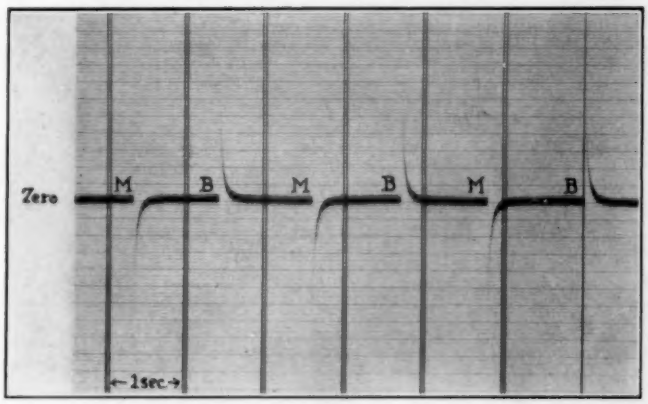


FIG. 1*b*

(Blinks: D. C. resistance of *Nitella*)

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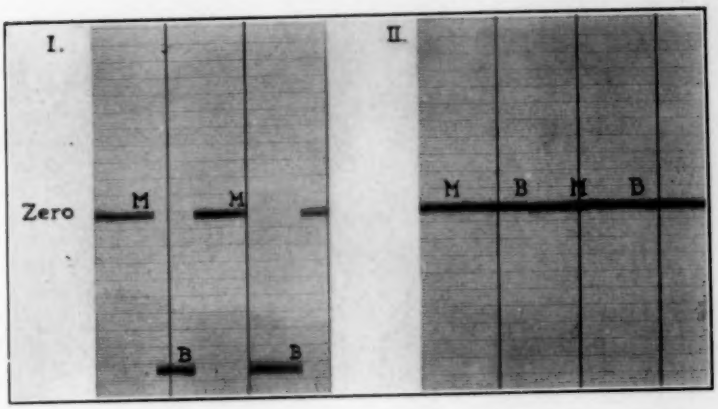

FIG. 1*c*

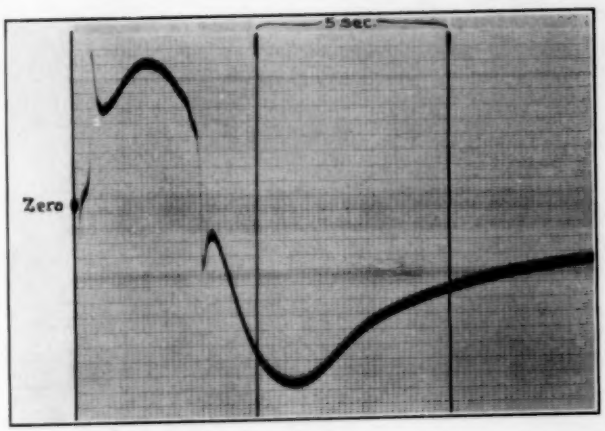

FIG. 1*d*

(Blinks: D. C. resistance of *Nitella*)

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THE EFFECT OF ISOELECTRIC AMINO ACIDS ON THE pH^+ OF A PHOSPHATE BUFFER SOLUTION

A Contribution in Support of the "Zwitter Ion" Hypothesis

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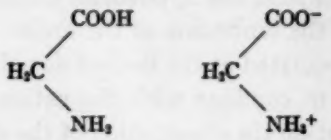
(Accepted for publication, February 1, 1930)

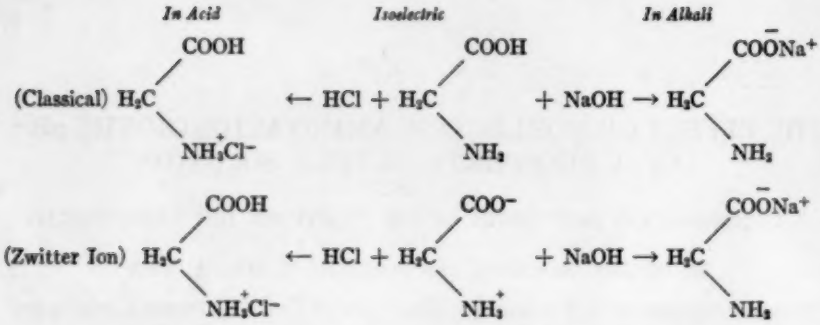
In recent years the conception in biochemistry of the ionization of amphoteric electrolytes developed by Walker (1) which is still generally accepted, has been gradually losing ground. The tendency has been to replace it with the hypothesis of Bredig (2), Adams (3) and Bjerrum (4), commonly designated as the "Zwitter Ion" hypothesis. The difference between the two hypotheses is represented by the following two formulae for isoelectric glycine.



I II

Formula I represents the current conception of the isoelectric condition of an amphoteric electrolyte. At the isoelectric point the molecule is not dissociated either as an acid or as a base (or the acid and basic dissociation are minimal and equal to each other). According to Formula II which represents the Zwitter Ion conception of isoelectric glycine, the molecule is, of course, also neutral, but this neutrality is due to the complete dissociation simultaneously of the acid and of the basic groups.

The changes which occur in acid and in alkali, according to the two conceptions, are represented by the following formulae.



As the above formulae show, there is no disagreement between the two hypotheses regarding the forms resulting from the addition of acid and of alkali. The difference is in the mechanism. According to the classical view, the effect of increasing alkalinity is to permit the ionization of the carboxyl group, which is so weak an acid that it dissociates only in alkaline reactions. Similarly the addition of acid permits ionization of the amino group, which is so weak a base that it can dissociate only in acid solutions. In alkaline solutions the buffering is due to the ionization of the weakly acid group, and in acid solutions to the ionization of the weakly basic group.

According to the Zwitter Ion hypothesis the effect of the addition of base is to depress the ionization of the amino group (both groups being completely dissociated at the isoelectric point), thus leaving the carboxyl group free to combine with the cation of the base added. Similarly in acid solution the dissociation of the carboxyl group is depressed, leaving the already ionized amino group free to form a salt with the acid. The buffering in alkaline solution is not due to the ionization of the acid group, but to the depression of the ionization of the amino group; and in acid solution to the depression of the dissociation of the acid group, rather than to the ionization of the amino group.

The essential difference between the two hypotheses consists in the strengths to be ascribed to the acid and basic groups. According to the older view the acid and basic groups are enormously weaker than acetic acid and ammonia, while according to the Zwitter Ion hypothesis they are each slightly stronger. The values are shown in Table I.

According to the Zwitter Ion hypothesis the titration constant obtained when glycine is titrated with acid is not the titration constant

(*i.e.*, the dissociation constant modified by the ionic strength of the solution) of the amino group, but the modified hydrolysis constant of the acid group. Similarly the titration constant obtained on titration with alkali is the modified hydrolysis constant of the amino group. In other words, according to Bjerrum:

$$K_{\text{acid}} = \frac{K_W}{K_b} \text{ and } K_{\text{base}} = \frac{K_W}{K_a}$$

where K_b and K_a are respectively the dissociation constants for base and acid according to the older view.

In support of the Zwitter Ion conception Bjerrum pointed out that the chemical structure of the amino acids does not warrant the very low values for the strengths of the acid and basic groups of the amino

TABLE I

The Dissociation Constants of Acetic Acid, Ammonia and Glycine

	K_a	K_b
Acetic acid	1.8×10^{-5}	—
Ammonia	—	1.9×10^{-5}
Glycine (classical values)	1.8×10^{-10}	2.6×10^{-12}
Glycine (Zwitter Ion values)	3.9×10^{-4}	5.6×10^{-3}

acids which are assigned to them on the older view. Indeed, as Lowry (5) pointed out, the effect of the amino group should be to increase the dissociability of the COOH group. From theoretical considerations it is impossible to explain an acid dissociation constant for glycine 100,000 times weaker than acetic acid, and a basic dissociation constant 10,000,000 times weaker than ammonia. On the other hand, the experimental values calculated according to the Zwitter Ion hypothesis can be derived from the constants for acetic acid and ammonia from the same theoretical considerations which permit calculation of the constants for the polybasic acids and polyacid bases.

For these reasons confidence in the older or classical view of the ionization of amphoteric electrolytes has diminished. The present feeling regarding the two theories is conveyed by the following quotation from Kirk and Schmidt (6):

"In choosing the classical system of nomenclature, we do not wish to imply that the above equations actually represent the true state of affairs in the solution. Since the proposal by Bjerrum of the 'Zwitter Ion' theory, it has become increasingly probable that this theory is a closer approach to the true status of an ampholyte in solution than is the classical theory."

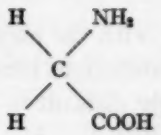
As the above quotation implies, the available evidence, favorable though it is, does not permit of a definite decision in favor of the "Zwitter Ion" theory as against the older view. All titration data can be absorbed equally well into either system. Until recently there has been no direct evidence obtained from experiments with amino acids themselves deciding in favor of either theory. It is believed that in the experiments described below this deficiency is supplied, and that the evidence which these experiments afford contributes toward a decision in favor of the "Zwitter Ion" theory.

Cohn (7) studied very thoroughly the effect of changing ionic strength upon the hydrogen ion concentration of mixtures of salts of primary and secondary phosphate solutions. His observations were that, for a given mixture of primary and secondary phosphates, the effect of increasing the ionic strength of the solution upon the activity coefficients of these two salts is to increase the hydrogen ion concentration, while decrease in the ionic strength results in a diminution of the hydrogen ion concentration. Robinson (8) observed the same effect of varying ionic strengths on the hydrogen ion concentrations of phosphate solutions when the phosphate concentration was kept constant and varying amounts of neutral salts were added.

It seems, then, that dilution of a phosphate solution whose pH was that of the isoelectric point of a given amino acid, with a solution of that amino acid at the pH of its isoelectric point might render a decision between the two theories. Since, according to the classical theory, an amino acid at its isoelectric point is dissociated neither as an acid nor as a base it may, in that condition, be compared to a non-electrolyte. Dilution of the phosphate solution with such a solution of an amino acid should change the hydrogen ion concentration of the phosphate solution in a manner similar to that effected by such non-electrolytes as urea or glucose, for example. These changes, as the experimental results below indicate, may be due, in part, to changes effected in the dielectric constant of the solutions.

On the other hand, since according to the "Zwitter Ion" hypothesis, at the isoelectric point both the acid and the basic group are completely dissociated, an amino acid molecule may, in solution in this form, be considered to resemble a strong electrolyte in the respect that it contributes to the ionic strength of the solution. However, since the ions of the isoelectric amino acid are not so free to move in solution as are the constituent ions of a strong electrolyte, it is to be expected that the effect of a given concentration of an isoelectric amino acid will be less than that of an equivalent concentration of a strong electrolyte, but much greater than that of neutral molecules such as glucose or urea. Assuming, therefore, an isoelectric amino acid, glycine, for example, to be dissociated as a monovalent electrolyte, and calculating its "ionic strength" on that basis, dilution of a phosphate solution with a solution of the amino acid of greater "ionic strength" should render the mixture more acid than the original phosphate solution, while dilution with a solution of lesser "ionic strength" should result in a less acid mixture. These changes will correspond in direction only to those obtained when the dilutions are carried out with solutions of similar ionic strengths of a strong electrolyte; but they will be distinctly greater than the effects obtained on dilution with neutral molecules.

Professor Pauling has pointed out to one of us that isoelectric glycine, even considered from the point of view of the classical conception, possesses a considerable electrical moment; and that more appropriate controls than glucose or urea would be compounds with electrical moments corresponding to that calculated for the molecule



From the values assigned to the NH_2 and COOH groups by Debye (9) the maximum electrical moment of isoelectric glycine, if it exists in the above form, is less than 1.9×10^{-18}. The additional controls chosen accordingly were ethyl alcohol, acetone, acetonitrile and phenol, with dipole moments of 1.7×10^{-18}, 2.7×10^{-18}, 3.6×10^{-18} and 1.7×10^{-18}, respectively. Phenol, whose acid dissociation constant is of the order of magnitude of 10^{-10}, at the hydrogen ion concentrations obtaining here, 10^{-6}, may be considered as a neutral molecule.

Two amino acids were employed, glycine and alanine. They were purified by recrystallization at their isoelectric points; and their purity was established by determinations of the relative and absolute values of total and free amino nitrogen. Solutions of these amino acids were made with ionic strengths, calculated on the basis of the "Zwitter Ion" hypothesis, equal to, greater than, and less than that of a phosphate solution. Dilution with amino acid solutions of the same ionic strength changed the hydrogen ion concentration very little. When the phosphate solution was diluted with an amino acid solution of greater ionic strength, the pH of the solution decreased; when the dilution was made with an amino acid solution of lesser ionic strength, the pH, of course, became greater. Similar results were obtained when the phosphate solution was diluted with solutions of potassium sulphate and of potassium chloride. On the other hand, when a phosphate solution was diluted with water or with solutions of glucose, ethyl alcohol, acetone, acetonitrile or urea, the pH of the resulting mixture was higher than that of the original phosphate solution. Phenol, on the other hand, induces an increase in acidity nearly as great as that due to glycine. It seems improbable that the similar effects of the amino acids and of phenol on the hydrogen ion concentration of a phosphate solution are due to the same cause, in the light of the divergent effects of ethyl alcohol, acetone, acetonitrile and urea, and also on account of the differences in structure and physical properties. Among the possible explanations which suggest themselves is that phenol in aqueous solution possesses a high degree of hydration.

The experimental results, with the exception of those with phenol, are in accordance with the predictions from the "Zwitter Ion" hypothesis, and are correspondingly difficult to interpret from the point of view of the classical theory.

The Zwitter Ion hypothesis receives very strong support from the observations made in the last few years on the dielectric constants of aqueous solutions of the amino acids. Hedestrand (10) found that the dielectric constants of solutions of glycine and of alanine increase with increasing concentrations of the amino acids, and that this increase is a linear function of the concentration of the amino acid. The dielectric constants of solutions of glycine and of alanine were found

also to be the same. Similar observations have been made by Walden and Werner (11) with alanine and betaine, by Fürth (12) with glycine, and by Blüh (13) with leucine. Aqueous solutions of alcohol, acetone, acetonitrile and glucose have markedly lower, and of urea slightly higher dielectric constants than that of water (14, 15).

Blüh, Walden and Werner, and Hedestrand consider the increased dielectric constants of aqueous solutions of the amino acids to be evidence in favor of their Zwitter Ionic constitution. In view of the present uncertainty in this field of the physical chemistry of solutions it is premature to consider this evidence alone as decisive. This increase in dielectric constant may be due simply to the increased concentration of molecules with a much higher dipole moment than that of water; or to the decomposition by the charges of the ionized poles of the amino acid of aggregates of water molecules whose dipole moment is low, into single molecules with higher electrical moment; or to the formation of large polarized complexes about each of the ionized poles of the amino acid, in the manner postulated for ions. On the other hand, the burden of explanation is definitely upon the classical theory as the observed effect of amino acids on the dielectric constants of aqueous solutions is in the expected direction only if they are considered as existing in solution as Zwitter Ions.

Recently methods have been devised for measuring the dielectric constants of relatively strong solutions of strong electrolytes (16). It has been found that at concentrations above 0.01 normal the values of the dielectric constants increase very quickly and at concentrations as low as 0.02 normal they considerably exceed that of water. In this respect also, therefore, the behavior of isoelectric amino acids is similar to that of strong electrolytes.

These observations on the effect of strong electrolytes strengthen the criticism of the theoretical foundations of the Debye-Hückel (17) activity theory, that the actual macroscopic dielectric constant of the solution should be employed in the equation for the activity of an ion instead of that of the pure solvent. The introduction later of the b term, and of the "salting out" term, both derived from the experimental data and both varying with each system, indicates this fundamental theoretical weakness in the theory, especially as even with the employment of these two "constants" the agreement between observed

and calculated values for activity coefficients over any considerable range of moderately high concentrations is only approximate.

The application of the Debye theory to solutions of weak electrolyte presents an even more complicated problem. With changing dielectric property of the solution, as occurs when the concentration of electrolyte changes, not only are variations induced in the activities of the ions, but also in the equilibrium between the ionized and unionized moieties, *i.e.*, in the equilibrium constant. Michaelis and Mizutani observed, for example, with increasing concentrations of alcohol, *i.e.*, with diminishing dielectric constant, that the pK′ (*i.e.*, the pH of a 50 per cent neutralized solution) of a weak acid progressively increases (20). If one attempts to calculate the pH in this case with such an equation as that of Cohn for aqueous phosphate solutions (7), taking into account only the effect of the changed dielectric constant on the activities of the ions, the calculation yields the reverse result, *i.e.*, the pH progressively diminishes with diminishing dielectric constant. The opposite result actually observed by Michaelis and Mizutani is due to the greater effect of the changing dielectric property of the solution on the dissociation equilibrium, than on the opposing effect on the activities of the ions.

In the case of the addition of an isoelectric amino acid to a phosphate buffer solution, it is not possible at present to determine how much of the observed decrease in pH is due to the resultant, of the effect of the changed dielectric property of the solution, of increasing the dissociation constant of the second hydrogen of phosphoric acid, on the one hand, and of its opposing effect of increasing the activities of the ions on the other; and how much to the increased ionic strength resulting from the addition of the amino acid. If, for whatever reason, the dissociation constant remains unchanged, then the whole responsibility for the decrease in pH of the phosphate amino acid mixture must be carried by the increase in ionic strength. Regardless of the extent to which these three factors contribute to the observed decrease in pH, the explanation can, it seems, be derived only from the conception of isoelectric amino acids as Zwitter Ions.

The effect postulated above of the changing dielectric constant of the solvent on the dissociation constants of weak acids, weak bases, and ampholytes, provides a qualitative explanation for some of the anom-

alous effects (*i.e.*, from the point of view of the Debye theory) of neutral salts on the ionization of weak electrolytes observed by Simms (24). Simms states that "the direction of all the deviations observed in this paper (with the exception of the effect of the SO_4 ions) is to render the solutions more acid than expected." Michaelis and Mizutani observed, as mentioned above, that in solutions of diminishing dielectric constant the dissociation of weak acids diminishes, whereas the dissociation of ammonia increases. The slope of the curves representing the acid constants is quite steep, while that of basic constants is nearly horizontal. Since, in the range of concentrations employed, increasing salt concentration increases the dielectric constant the predictable effect of the addition of strong electrolytes, extrapolating the curves into the zone of increasing dielectric constants, is to render a solution of a weak acid more acid, and a solution of a weak base slightly less basic, *i.e.*, more acid. In the case of an amphoteric electrolyte, such as glycine, the effect of addition of salt is to increase the acid dissociation constant considerably, and to depress slightly the basic dissociation constant. Both effects tend to move the isoelectric point of the amino acid toward the acid side. This prediction is confirmed by the observation of Simms that "isoelectric points drop with increase in ionic strength." A similar explanation may account for the gradually increasing values with increasing concentration, of the dissociation constants of such moderately strong acids as the first hydrogen of phosphoric acid and of hydrosulfate, observed by Sherrill and Noyes (25).

It is important that the similarity in behavior of isoelectric amino acids to strong electrolytes, which is predictable from the Zwitter Ion and not from the classical conception, is independent of theoretical considerations of electrolyte solutions. While the Debye theory accounts qualitatively for the experimental facts, the explanation of the quantitative differences between amino acids and strong electrolytes, and between different non-electrolytes (Table IX) requires a development of the theory which has not yet been attained.

Professor Pauling has pointed out that whatever be the electrical moments of the amino acids, the moment of a protein molecule is probably low, on account of the probable even distribution of free carboxyl and amino groups about the periphery of the large molecule. This prediction is supported by the findings that the dielectric con-

stants of aqueous solutions of albumin and of gelatine are distinctly lower than that of water (17). These determinations of the dielectric constants of the amino acids and proteins cannot be accepted without reservation, because, apparently, no precaution was taken to ensure that the substances employed were in their isoelectric condition and that the solutions were salt-free. It is probable that they were all more or less in the salt form. However, in the case of the amino acids, the increase in dielectric constant is so large, and in the case of the proteins the decrease is so great, that it is unlikely, if the determinations were made with these ampholytes in isoelectric, salt-free solutions, that the direction, or even the magnitude of the values would be significantly different. The magnitude of the dissociation constants of the free carboxyl and free amino groups on the periphery of the protein molecule would, of course, still be of the order of magnitude of acetic acid and ammonia.

On account of these variations in the dielectric constants of aqueous solutions of electrolytes, amino acids and proteins, such quantities as dissociation constants, and activity coefficients, determined in simple aqueous solutions, may, it seems, be employed without modification in considerations of physiological conditions, only very tentatively, until more data are available.

The work of Pauli and his co-workers (18) on the behavior of electrolyte-free proteins supports the Zwitter Ion hypothesis and suggests that it may be significant in the explanation of protein behavior *in vivo*. Pauli and his collaborators found that albumin may behave as a cation in hydrogen ion concentrations considerably removed from the isoelectric point. This behavior, and certain observations on the conductivity of serum albumin solutions saturated with CO_2 were best accounted for by assuming the existence of Zwitter Ionic protein. By a similar explanation Pauli and his collaborators accounted for the behavior of electrolyte-free protein toward neutral salts and strong acid.

The values of the dissociation constants of the substitution compounds of acetic acid are also in better accord with the Zwitter Ion hypothesis.

Lowry, quoting the observations of Vorländer on the strength of the carboxyl groups in the substituted anilino-acetic acids, points out

that the amino group is an acylous substituent, *i.e.*, that its effect is to increase the strength of an adjacent acid group. Table XI shows that this effect of the amino group on the COOH group in glycine is demonstrable only when its value is calculated from the point of view of the Zwitter Ion hypothesis. The classical theory gives an anomalous, very low value for the COOH group of glycine and forces upon the amino group the property of markedly weakening the acid group. Similar evidence is contained in the values for the acid and basic constants of the substituted amino acids. Whereas the negative logarithm of the acid dissociation constant of glycine according to the classical theory is 9.75, this value for acetylaminoacetic acid is 3.64, and for anilino-acetic acid 4.4. The classical value for the negative logarithm of the basic dissociation constant of glycine is 11.59, for amino-acetic acid ethyl ester it is 7.01 (19).

Michaelis and Mizutani (20) found that the dissociable groups of glycine titrated in acid solution varied, with increasing alcohol content, in the same manner as COOH groups, while the constant titrated in alkaline solution behaved like ammonia. Michaelis and Mizutani wrote "Dieser Befund steht in schöner Übereinstimmung mit der neueren Auffassung von der Dissoziation des Glykokoll."

Experimental Procedure and Results

The glycine used was recrystallized twice from an aqueous solution at pH 6.0. After drying over sulfuric acid to constant weight the hydrogen ion concentration of a 3 per cent solution in CO_2-free water was at pH 6.27. The isoelectric zone of glycine extends from approximately pH 4.5 to pH 7.5, and its isoelectric point is at pH 6.08 (6). The close proximity of the hydrogen ion concentration of the solution of glycine in water to that of its isoelectric point was taken to indicate that no significant amount of the amino acid was present in the form of a salt. The free amino nitrogen corresponded to 18.96 per cent of the weight of the glycine. The theoretical per cent of total nitrogen and free amino nitrogen in glycine is 18.65 per cent.

The alanine used was twice recrystallized. The pH of a 3.56 per cent solution in CO_2-free water was 6.09. The isoelectric point of alanine is at pH 6.04; the isoelectric zone extends, as in the case of glycine, from pH 4.5 to pH 7.5. The free amino nitrogen corresponded to 15.61 per cent of the weight of the dissolved alanine. The theoretical per cent of total and free amino nitrogen is 15.70.

The phosphate solutions were prepared from KH_2PO_4 and K_2HPO_4 according to the data of Cohn. It was not deemed necessary to obtain the phosphate salts in the same degree of purity as the amino acids as the results to be obtained were

to consist of differences in hydrogen ion concentration before and after dilution. It is probable that they were not perfectly dry and that the K_2HPO_4 salt contained a small amount of impurities. The hydrogen ion concentrations of the phosphate mixture do not coincide absolutely with, but are close to, the values obtained by Cohn. 0.5 molar solutions of the two salts were prepared. These were diluted as required. In a later series of experiments mixtures of KH_2PO_4 and Na_2HPO_4 were employed. Solutions with a molecular ratio of 4 of the acid to the basic salts were used. The hydrogen ion concentrations which were obtained were very close to the theoretical isoelectric point of glycine.

Two preparations of glucose were employed, one a recrystallized specimen, the other Pfanstiehl's C.P. anhydrous d-glucose, with an ash content of 0.05 per cent. The aqueous solutions of both samples were neutral. The phenol was a Merck "blue label" preparation, and the urea Baker's analyzed; the ethyl alcohol and acetone had been redistilled, the acetonitrile was a specimen obtained from Professor Lucas. The strong electrolytes employed were a pure sample of potassium sulfate, and Merck's "blue label" potassium chloride. The determinations of the hydrogen ion concentration were carried out electrometrically with a hydrogen electrode and a saturated calomel half cell. A Leeds and Northrup hydrogen ion potentiometer was employed and the error for any one observation was not more than 0.0002 volts. Observations were repeated on each solution with change of gas for each reading until three consecutive pH readings were obtained whose difference was not greater than 0.0002. The type of hydrogen electrode employed was that devised by Moloney (21). With this apparatus determinations made on the same solution at different times coincided within the limits of ± 0.01 pH.

No attempt was made in the earlier experiments to carry out the observations at constant temperature. Observations of the temperature of the calomel half cell and of the solution were made for each reading. All the readings were then standardized by deducting the E.M.F. of the saturated calomel half cell from the E.M.F. observed. The resulting value, which was the E.M.F. of the solution, was corrected for temperature by calculating the corresponding E.M.F. at 25°C. To this value was added the E.M.F. of the saturated calomel electrode at 25°C. The pH was then calculated from this value. The values for the calomel cell are those obtained by Michaelis, quoted by Clark (22).

The experiments in which the effects were compared, of compounds with known electrical moments with those due to isoelectric glycine, were performed later with different preparations of the phosphate salts, and recrystallized glycine. The measurements of the hydrogen ion concentration were made with a Leeds and Northrup Type K potentiometer, in an air bath maintained at 25°C. The duplicate readings here agreed to within 0.0001 volts; and each of the values given represents concordant duplicate observations with each of two electrodes.

If isoelectric glycine in water is in the form of a Zwitter Ion, dilution of the phosphate solution with a glycine solution of greater ionic

TABLE II

The Effect of Dilution of a Phosphate Solution with Isoelectric Glycine, Potassium Chloride, and Water

Final ionic strength of phosphate solution	pH^+ in various solvents		
	Water	0.25 molar isoelectric glycine	0.25 molar KCl
0.087	6.25	6.08	6.01
0.022	6.38	6.20	6.04
0.0055	6.49	6.26	6.05

TABLE III

The Effect of Dilution of a Phosphate Solution with Solutions of Isoelectric Glycine of Greater "Ionic Strengths"

Phosphate solution		Glycine solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	"Ionic strength"		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	°C.	°C.	
30	0.1	—	—	0.6489	23	23	6.85
10	0.1	20	0.4	0.6451	25.6	25.8	6.74
10	0.1	20	0.3	0.6482	25.6	24.5	6.83
10	0.1	20	0.2	0.6507	25.6	25.5	6.84
10	0.1	20	0.1	0.6512	25.6	25.5	6.84
10	0.1	20	water	0.6560	25.6	24.5	6.96

TABLE IV

The Effect of Dilution of a Phosphate Solution with Solutions of Isoelectric Glycine of Lesser "Ionic Strengths"

Phosphate solution		Glycine solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	"Ionic strength"		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	°C.	°C.	
30	0.4	—	—	0.6391	25	24.5	6.67
10	0.4	20	0.4	0.6379	25	23.5	6.66
10	0.4	20	0.3	0.6413	25	24.5	6.70
10	0.4	20	0.2	0.6433	25	24.8	6.73
10	0.4	20	0.1	0.6451	25	24.5	6.77
10	0.4	20	water	0.6473	25	25.0	6.79

strength may be expected to cause a decrease in the pH of the mixture, while dilution with a solution of lower ionic strength may be expected to increase the pH. The results of such an experiment are shown in Tables II, III and IV.

TABLE V

The Effect of Dilution of a Phosphate Solution with Solutions of Isoelectric Alanine of Greater and Lesser "Ionic Strengths"

Phosphate solution		Alanine solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	Ionic strength		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	*°C.*	*°C.*	
10	0.4	20	0.4	0.6362	19.0	19.0	6.68
10	0.4	20	0.2	0.6407	20.25	21.5	6.71
10	0.4	20	0.08	0.6418	20.5	21.5	6.73
10	0.4	20	water	0.6398	18.75	18.6	6.75
5	0.4	25	water	0.6421	18.5	19.25	6.77
15	0.16	35	0.36	0.6361	20.25	21.25	6.63
15	0.16	35	0.26	0.6389	20.5	21.25	6.69
15	0.16	35	0.09	0.6412	20.5	21.0	6.73
15	0.16	35	water	0.6460	20.5	20.25	6.83

TABLE VI

The Effect of Dilution of a Phosphate Solution with Solutions of Potassium Sulfate of Various Ionic Strengths

Phosphate solution = 0.4	Potassium sulphate solution		E.M.F. observed	Temperature		pH
	Volume	Ionic strength		Of calomel half cell	Of solution	
cc.	*cc.*		*volts*	*°C.*	*°C.*	
30	—	—	0.6402	23.5	23.9	6.66
10	20	0.4	0.6380	23.0	23.8	6.65
10	20	0.3	0.6391	22.25	23.35	6.66
10	20	0.2	0.6399	22.5	22.5	6.70
10	20	0.1	0.6436	22.5	23.2	6.75
10	20	water	0.6462	22.0	22.7	6.80

Tables II and III show that dilution of a phosphate solution with a glycine solution of greater ionic strength causes an increase in the hydrogen ion concentration. Dilution with a glycine solution of

lower ionic strength, Table IV, brings about a decrease in the hydrogen ion concentration.

Similar results were obtained when a phosphate solution was diluted with an isoelectric solution of alanine. These results are given in Table V.

Dilution of the phosphate solution with solutions of potassium sulfate similar to those of the amino acid solutions used, gave similar changes in hydrogen ion concentration. This is shown in Tables VI and VII.

TABLE VII

The Effects of Dilution of a Phosphate Solution with Potassium Chloride Solutions of the Same, Lesser, and Greater Ionic Strengths

Phosphate solution		Potassium chloride solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	Ionic strength		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	*°C.*	*°C.*	
30	0.4	—	—	0.6391	25	24.5	6.67
5	0.4	25	0.4	0.6368	26.5	25.5	6.62
10	0.4	20	0.4	0.6382	26.4	25.0	6.65
15	0.4	15	0.4	0.6395	26.0	25.5	6.66
20	0.4	10	0.4	0.6400	26.6	26.0	6.66
10	0.4	20	0.1	0.6459	25.7	26.2	6.75
10	0.4	20	0.2	0.6426	26.0	26.0	6.70
10	0.4	20	0.3	0.6402	26.2	25.8	6.67
10	0.4	20	0.4	0.6382	26.4	25.0	6.65
10	0.4	20	0.6	0.6355	27.2	26.5	6.58
10	0.4	20	0.8	0.6330	26.7	26.0	6.54

Table VIII shows the effect of dilution of a solution of phosphate with a glucose solution whose "ionic strength" was calculated on the same basis as that of the amino acid solutions. The magnitude of the change, however, is less than that obtained with water, and depends to some extent upon the concentration of glucose. Randall and Failey (23) found that the activity coefficients of non-electrolytes are affected by the presence of electrolytes. As the results in Tables VIII and IX show, the activity of electrolytes is affected by the presence of non-electrolytes. This phenomenon, nevertheless, is not

TABLE VIII

The Effect of Dilution of a Phosphate Solution with a Glucose Solution

Phosphate solution		Glucose solution		E.M.F.	Temperature		pH
Volume	Ionic strength	Volume	Mols. per litre		Of calomel half cell	Of solution	
cc.		*cc.*		*volts*	°C.	°C.	
30	0.4	—	—	0.6391	25	24.5	6.67
25	0.4	5	0.4	0.6372	21.8	21.2	6.68
20	0.4	10	0.4	0.6371	21.8	20.0	6.70
15	0.4	15	0.4	0.6398	22	21.2	6.72
10	0.4	20	0.4	0.6405	22	20.5	6.75
5	0.4	25	0.4	0.6413	21.7	20.6	6.76
15	0.4	15	water	0.6421	22.5	21.8	6.75
5	0.4	25	water	0.6465	22.0	21.5	6.83
30	0.1	—	—	0.6489	23.0	23	6.85
15	0.1	15	0.4	0.6493	22.5	22.5	6.86
10	0.1	20	0.4	0.6503	22.3	22.0	6.88
5	0.1	25	0.4	0.6525	22.5	22.0	6.92

TABLE IX

The Effects of Glycine, Ethyl Alcohol, Acetone, Acetonitrile, Urea and Potassium Chloride on the C_{H^+} of a Phosphate Solution

Substance dissolved in phosphate solution	Electrical moment of substance dissolved $\times 10^{18}$	Dielectric constant of the aqueous solution of substance dissolved	pH
0.0165 Molar Phosphate alone	—	—	6.35
Ethyl alcohol 1 Molar	1.63–1.74	50 (ca.)	6.43
Acetone 1 Molar	2.63–2.97	45 (ca.)	6.51
Acetonitrile 1 Molar	3.4–3.94	100 per cent Acetonitrile 36 (19)	6.84
Urea 1 Molar	—	82 (ca.)	6.37
Isoelectric glycine 1 Molar	Calculated for classical structure 1.85	104	6.05
Potassium chloride 1 Molar	—	—	5.84

significant here. The results obtained on dilution with glucose are slight compared to those obtained with glycine and alanine.

In Table IX are recorded the hydrogen ion concentrations of 0.0165 molar phosphate ($\mu = 0.022$) solutions containing in molar concentra-

TABLE X

The Effects of Potassium Chloride, Isoelectric Glycine, and Phenol on the C_{H+} of a Phosphate Solution

Substance dissolved in 0.0165 molar phosphate $\mu = .022$	Molar concentration of substance dissolved	pH
0.0165 molar phosphate alone	—	6.35
KCl	0.2	6.09
	0.5	5.96
	1.0	5.84
Isoelectric glycine	0.2	6.26
	0.4	6.17
	0.6	6.14
	0.8	6.09
	1.0	6.05
Phenol	0.2	6.24
	0.4	6.27
	1.0	6.11

TABLE XI

Dissociation Constants of Acetic Acid and of Some of Its Substitution Compounds

Substance	pK_a
$H\text{-}CH_2COOH$	4.74
$COOCH_3\text{-}CH_2\text{-}COOH$	4.34
$O{=}CH\text{-}COOH$	3.74
$NO_2\text{-}CH_2\text{-}COOH$	3.34
$NH_2\text{-}CH_2\text{-}COOH$	9.75 Classical value 3.41 Zwitter Ion value

tion, isoelectric glycine, ethyl alcohol, acetone, acetonitrile and urea. As we were concerned here with directional changes only no corrections are introduced for the vapor tensions of the ethyl alcohol, ace-

tone or acetonitrile. In every case the direction of the change indicated electrometrically was confirmed by a colorimetric determination.

These results in Table IX show the different effect of glycine on the hydrogen ion concentration of a phosphate solution from that of substances with electrical moment corresponding to that calculated for the classical form of isoelectric glycine.

Tables II and X show the comparative effects on the hydrogen ion concentration of a phosphate solution of potassium chloride, isoelectric glycine, and phenol. The behavior of phenol as mentioned above may be due to its high degree of hydration; but in the absence of data it constitutes a definite flaw in the evidence.

SUMMARY

The relative merits of the classical conception and of the Zwitter Ion conception of the dissociation of amphoteric electrolytes are discussed, and the following data are presented which confirm the Zwitter Ion hypothesis of Bjerrum, and which are not in accord with the classical view.

1. Amino acids in the isoelectric form resemble strong electrolytes in that they contribute to the ionic strength of the solution.

2. The dielectric constants of aqueous solutions of amino acids, like those of solutions of strong electrolytes greater than 0.02 normal, are considerably greater than that of pure water.

3. The magnitude of the dissociation constants of substituted acetic acids and of glycine, are more easily accounted for with the Zwitter Ion than with the classical conception.

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THE INFLUENCE OF THE QUANTITY OF NUTRITION UPON THE GROWTH OF THE SUCKLING MOUSE

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The contrast between the character of the growth curves of the embryo and the suckling mouse demands an explanation. Before birth the curve is a parabola (MacDowell, Allen and MacDowell '27). After birth some of the published averages show a slight concave phase, but this becomes convex by the end of the first week. That this difference might be due to some external condition rather than to internal changes was suggested by the fact that the parabolic curve of the embryo guinea pig continues uninterrupted through the stages of development corresponding to those of the suckling mouse (MacDowell, Allen and MacDowell '27). While a constant environment is maintained (prenatal guinea pig) growth continues to follow the same curve; when a new environment is entered (suckling mouse) the character of the curve is changed. After birth, new uses of energy and a new source of nutrition will account for a general modification in the curve as a whole, but not for the convexity normally found in the second week. The influence of special diets upon growth is well known. The vitamine diets of Osborne and Mendel '26 produce astonishing accelerations in the growth of the rat, which Brody '28 points out change the constants but not the nature of the mathematical formula for the growth curve. In contrast to this, in the suckling period of the mouse, by increasing the quantity of mother's milk available, we now find an equally surprising acceleration in growth, which continues the concave curve of the first week throughout the second week up to the beginning of natural weaning.

* Experiments all carried out at Cold Spring Harbor, 1927–29; W. H. Gates in active cooperation summer 1928; assistance of Jean M. Marsh and T. Lanes is acknowledged.

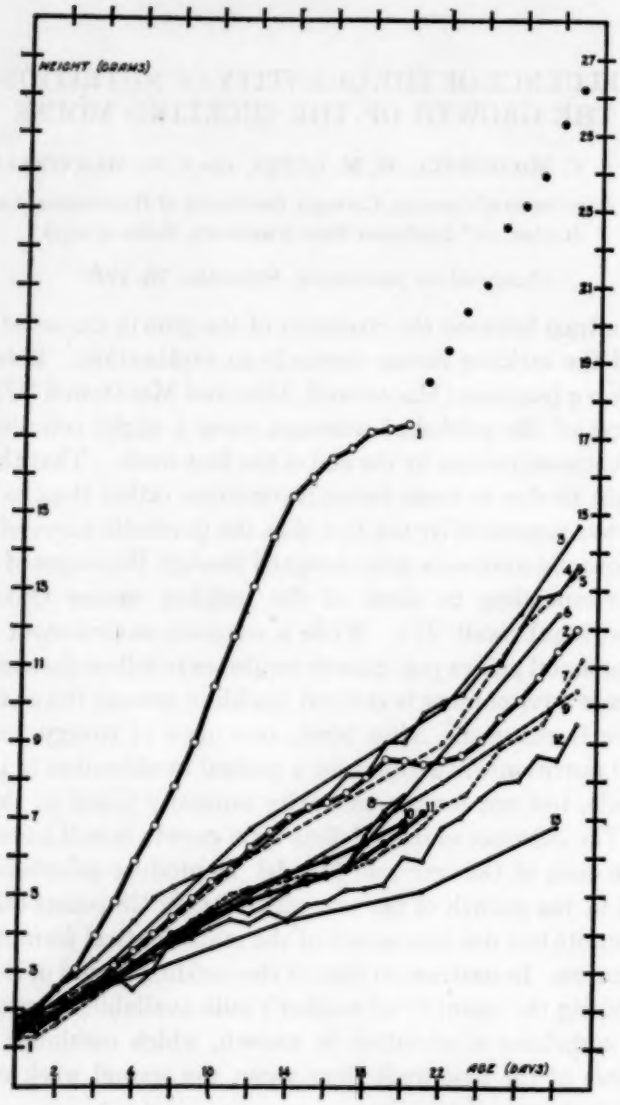

FIG. 1. Published growth curves for the suckling mouse, compared with the curve that can be obtained by increasing the quantity of mother's milk.

Curve 1: Six heaviest females at 14 days in present experiments; number in litter reduced to four at birth and later to one (for details see legend for Fig. 4); beyond 20 days this curve is continued by unconnected points giving the weights of a single one of the six.

Eleven series of published average weights for the suckling mouse are given in Fig. 1, Curves 3 to 13. Considering the great range in material, methods, and laboratories, these curves are fairly consistent. Birth weights are similar; at 14 days the range in weights is 4 to 7 gm. In the first week, as already mentioned, several curves show a tendency toward a concave form; the second week begins with a break which leads to a marked depression in the third week. An acceleration in the fourth week frequently gives a steeper slope than before the depression. This rise, and the preceding depression, form the chief features of the curve that support the theory of growth cycles as formulated by Robertson ('16) ('26), who claims the depression has nothing to do with weaning, since the mother is not removed until a week after the depression begins. Rather, he says, the slowing down represents the end of the dominance of one chemical reaction, while the subsequent acceleration is the beginning of the dominance of a more rapid, independent chemical reaction.

Experiments to Increase the Available Mother's Milk

In the attempt to determine the form of the growth curve of the suckling mouse when the quantity of food is not a limiting factor, several series of experiments have been carried out. Since a maximum

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FIG. 1—*continued.*

Curve 2: Six control females from the same strain: mothers fed and cared for as those for Curve 1, but no reduction in number of young.

Curve 3: from Thompson and Mendel '18; 15 males.

Curve 4: from Saller '27; 3 males.

Curve 5: from Judson '16; 20 males.

Curve 6: from Robertson '16; sex not given: numbers range from 24 to 65, no two successive days include same number of mice.

Curve 7: from Ostwald '08: seven mice, sex not given.

Curve 8: from Robertson and Cutler '16; sex not given, numbers range from 18 to 48; great irregularity from day to day.

Curve 9: Davenport and Swingle '27; litters of "7 to 5," males and females: after birth day, numbers range from 102 to 123.

Curve 10: from Gates '25; males and females: numbers decline from 678 to 471.

Curve 11: from Parkes '26; males and females; 407 mice; unweighted averages of averages per size of litter of averages per litter.

Curve 12: from Robertson and Delprat '17; sex not given; numbers irregular, 118 to 60.

Curve 13: Stieve '23; 30 males.

of milk is being sought, a detailed account of the stages by which the technique has developed is not pertinent. The primary item in this technique consists of reducing the number of young nursed by one mother. Studies have been made on the amount and the time of this reduction; on the use of mouse and rat foster mothers under different combinations of age and recency of parturition of the mother; and on the influence of the ovaries on the milk supply. The conclusions reached by these studies may be summarized as follows: reduction to four at birth, to two when 3 days old, and to one when 5 days old has produced our maximum growth; reduction to less than four at birth is a disadvantage. Foster mothers whose young were born 5 days after the young in question may do as well, more often less well, but not better than the own mothers; alternating foster mothers in 12 hour shifts does not increase the available milk; rats make good foster mothers for mice, but not as good as own mothers (neither in this case nor in any other has an unhealthy condition been found as was reported by Parkes '29 as a result of the rapid growth obtained by rat foster mothers); the break in the curve at 15 days is not due to a depression of lactation due to returning oestrus, since spaying mothers at parturition does not modify the break; the independence of the 15 day break and the quantity of milk the mother can produce is shown by the fact that a mother 18 days after parturition can raise four new born young, giving them a larger total amount of milk than was given her own (reduced) litter.

The mothers' food was the regular diet used for the whole colony: fresh milk, Spratt's cod liver oil dog bread soaked in water, oats, hemp and canary seed; this was replenished twice a day in these experiments. The boxes were cleaned daily; the temperature was controlled at approximately 79°F. Daily weighings were made on a Sartorius balance. The mice came from the Bagg albino strain, which has been inbred brother by sister in this laboratory since 1922.

Immediate Influence of the Mother on the Curve of the Suckling Young

The immediate dependence of the growth curve upon the milk supply (mother) rather than upon the young mouse itself is strikingly emphasized by two special series of experiments. In the first of these the growth of suckling mice from another strain (B) was

found to differ from that of mice in the strain (A) used in all other experiments. Fig. 2 gives the averages of 8 mice from Strain A and

FIG. 2. Difference in growth curves of two strains that depends upon mothers and not upon young themselves.

Curve 1: Solid line; averages of 8 Bagg albino young (2 ♂, 6 ♀) nursed by own mothers with three litter mates for 5 days, then two of these removed; left alone with mother from the 9th day; mothers removed at end of third week.

Curve 2: Broken line; averages of 5 young from Storrs-Little strain (1 ♂, 4 ♀) nursed by own mothers; reductions and removal of mothers as in Curve 1.

Curve 3: Dotted line; averages of 6 young from Storrs-Little strain (1 ♂, 5 ♀), fostered on day of birth by Bagg albino mothers whose own young were born on the same day; each mouse nursed with three litter mates for 4 days, two removed on the fifth day, and the third on the eighth day; mothers removed after 17 days.

5 mice from Strain B. The mice in Strain B did not grow as fast in the first week; they started on the rapid post weaning rate before the mothers were removed at the end of the third week. In Strain A there was frequently a loss for a day or two after the mothers were removed, before the rapid rate was resumed. This difference in the

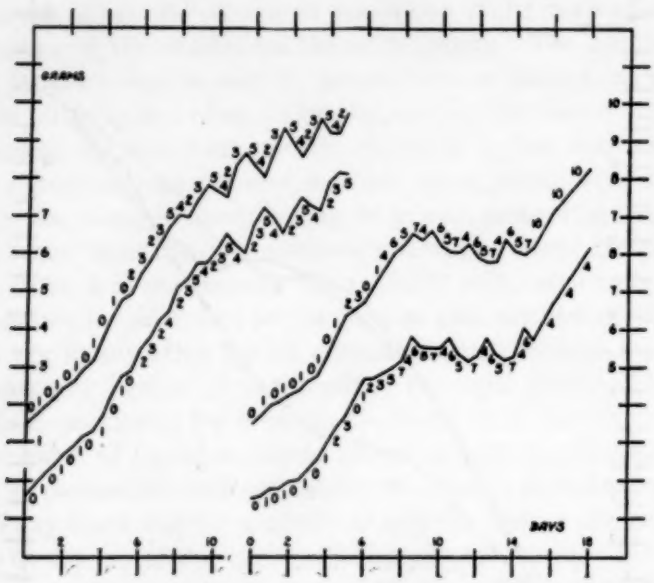

FIG. 3. Two pairs of curves of individual mice fostered by a succession of mothers in 12 hour shifts, showing the dependence of the growth of the young on the mother. Each litter, reduced to four at birth, was separated into pairs after 4 days; and after 7 days each mouse was nursed separately, each foster mother nursing alternately two mice from the same litter. Young weighed at each change of nurses. Numbers along the lines indicate which nurse was on duty; 0 = own mother. Foster mothers first on duty day after parturition, 12 hours after removal of own young. Note that the zero point for weight for the lower curve of each pair is on the base line; the upper curves start 2 units higher.

curves was shown to be due to the mothers and not to any genetic difference in the young themselves, by a set of young from Strain B that were fostered by mothers from Strain A. The difference was entirely eliminated. The mothers in Strain B were not as good as the mothers in Strain A: they did not give as much milk in the first week and natural weaning was completed sooner.

In the second series the complete dependence of the rate of growth on the mother was even more strikingly demonstrated by giving a suckling mouse, in 12 hour shifts, to a series of foster mothers which were not nursed in alternate shifts. The experiments were undertaken in the hope of providing a more bountiful milk supply, but in most cases the foster mothers did poorly. However a few mothers did well under these conditions and a series was obtained showing the results of good and poor nurses in alternate shifts (see Fig. 3). As the weights were taken every 12 hours the curves show the changes in weight for which each mother was responsible. The striking point is that the pairs of curves parallel each other; one mother gives a good gain each time on duty, another gives a loss; the same succession of mothers gives each young practically the same series of gains and losses. The numbers along the curves indicate which foster mother was on duty.

Maximum Curves

The average weights of the six females, in all our experiments, that were the heaviest on the 14th day are plotted in Fig. 1, Curve 1. Beyond the 20th day the curve is continued by the weights of only one of the six. Individual data for these six are given in Table I and Fig. 4. To show that the difference between these and the published curves does not depend upon the particular strain of mice used, nor upon the diet of the mothers, nor upon the methods of feeding and caring for them, the averages are given in Fig. 1, Curve 2, of six females from the same strain, raised on mothers handled and fed in the same way as the others, but without reduction in the number of young. These controls are seen to agree closely with the higher published curves. A further check is given by the averages of mice from the same strain raised under the normal routine of the whole colony; 17 females 22 days old gave an average of 8.59 gm.; 24 other females, 23 days old, averaged 9.30 gm. These points lie between Curves 2 and 6 in Fig. 1.

At 14 days the published averages and the controls show less than half the weight attainable at this time. But more important than the difference in absolute weight is the change in the shape of the curve accompanying the removal of external limitation—a continued upward sweep of the original concave curve lasting until the end of the

second week and terminated by a sudden and continued break. These mice are considered to approach the limit of growth capacity under the general conditions of these experiments. There is no claim that they

TABLE I

Individual Weights (Grams) of the Six Females That Reached the Highest Weight on the 14th Day

Age	G59,2	GS2,3	GS3,4	G108,0	G109,3	G116,0
Birth	1.46	1.39	1.55	1.62	1.61	1.54
1	1.83	1.81	2.01	1.98	2.04	2.08
2	2.37	2.34	2.61	2.52	2.67	2.71
3	3.12	2.92	3.16	3.23	3.20	3.27
4	3.84	3.56	3.93	4.02	4.06	4.19
5	4.66	4.30	4.72	5.02	4.90	5.31
6	5.77	5.38	5.80	6.19	5.89	6.18
7	6.75	6.25	6.84	7.16	6.96	6.79
8	7.82	7.13	7.93	8.17	8.09	7.79
9	8.78	8.44	9.19	9.49	9.16	8.78
10	10.29	10.06	10.54	10.74	10.47	10.47
11	11.58	11.32	12.05	11.90	11.77	11.92
12	13.01	12.71	13.35	13.08	12.70	13.45
13	14.34	14.15	14.80	14.20	14.13	14.60
14	15.53	15.34	15.60	15.28	15.15	15.54
15	16.06	15.64	16.32	16.05	15.79	15.72
16	16.49	16.06	16.57	16.37	16.49	16.52
17	16.58	16.27	17.03	16.83	16.80	16.66
18	17.21	17.09	17.36	17.14	17.28	16.69
19	17.62	17.32	16.59	17.52	17.49	16.78
20	17.72	18.04	16.31	17.68	17.68	16.73
21	17.16	18.48	15.94	17.95	17.59	16.72
22	17.02	19.30	16.18	18.44	17.54	17.40
23	16.32	20.34	16.83	18.96	17.66	17.94
24	15.80	21.04	17.65	19.77	17.96	18.42
25	15.25	22.56	18.56	20.66	18.26	18.89
26	16.00	23.12	18.69	21.46	18.35	19.00
27	15.80	23.92	20.37		18.51	
28	16.23	25.30	20.08		18.90	
29	17.00	26.21	21.55		19.66	
30	17.82	26.47	21.43		19.67	

have actually reached such a point. In many cases the curves of the individual mice show almost mathematical smoothness from day to day, especially in the early part (see Figs. 4 and 5). But at the rate

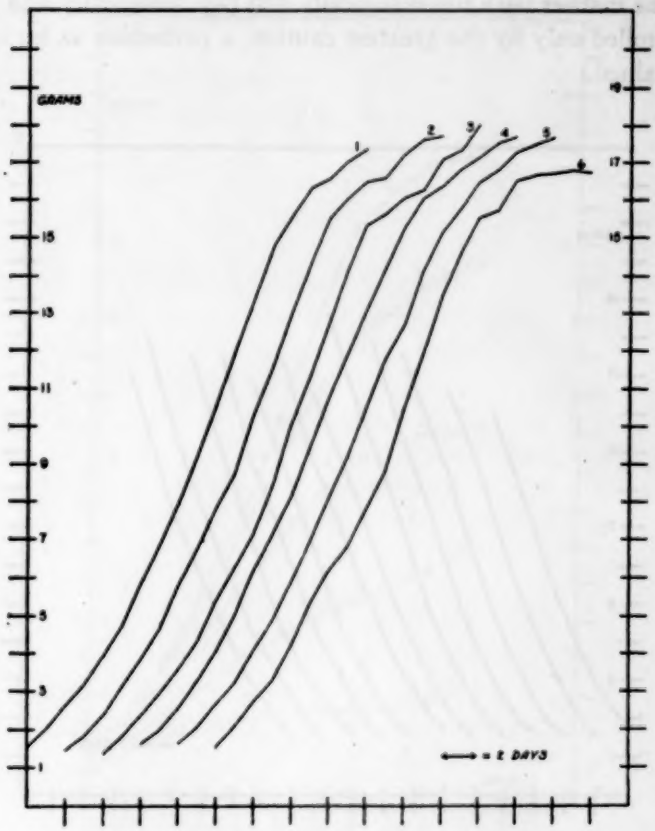

FIG. 4. Individual curves for the six females averaged in Fig. 1, Curve 1.

Curve 1: (GS3, ♀4) born in litter of 11; reduced to four at birth and both ovaries removed from mother; in 5 days three litter mates removed.

Curve 2: (G59, ♀2) born in litter of 9, reduced to four at birth; in 5 days this mouse was given to a foster mother whose litter had just been born and discarded.

Curve 3: (GS2, ♀3) born in litter of 8, reduced to four at birth and both ovaries removed from the mother; in 5 days other three litter mates removed.

Curve 4: (G108, ♀0) born in litter of 10, reduced to four at birth, in 3 days two more discarded, and in 5 days after birth left alone with mother.

Curve 5: (G109, ♀3) born in litter of 9; reductions as in Curve 4.

Curve 6: (G116, ♀0) born in litter of 7; reductions as in Curve 4.

obtained, growth is influenced by subtle changes in conditions that affect the mother both physiologically and psychologically and are to be controlled only by the greatest caution, a perfection so far clearly not attained.

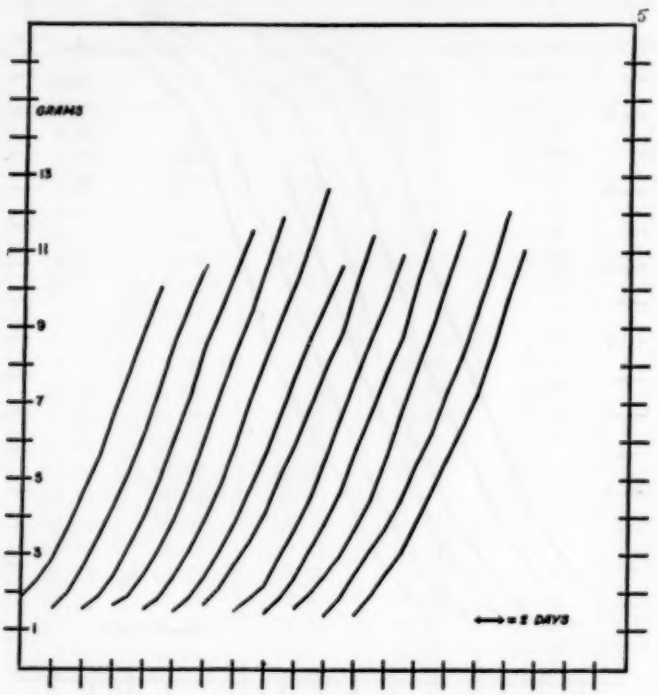

FIG. 5. Individual curves for twelve mice showing the consistency of slope and weight and the smoothness possible for individuals. These curves show that the second week can be entered without either an inflection or a temporary acceleration sometimes found the day after the litter is reduced to a single mouse. These curves are taken from various experiments; they broke at different times before the end of the second week.

The change in shape of the curve as the maximum is approached is illustrated in Fig. 6. Curve 4 (Gates '25) is based on over 600 mice from different strains raised in this laboratory in 1922. Of these 600 the six that were the lightest at 14 days are averaged in Curve 5; the heaviest six at 14 days, in Curve 3. Curve 2 gives the averages of

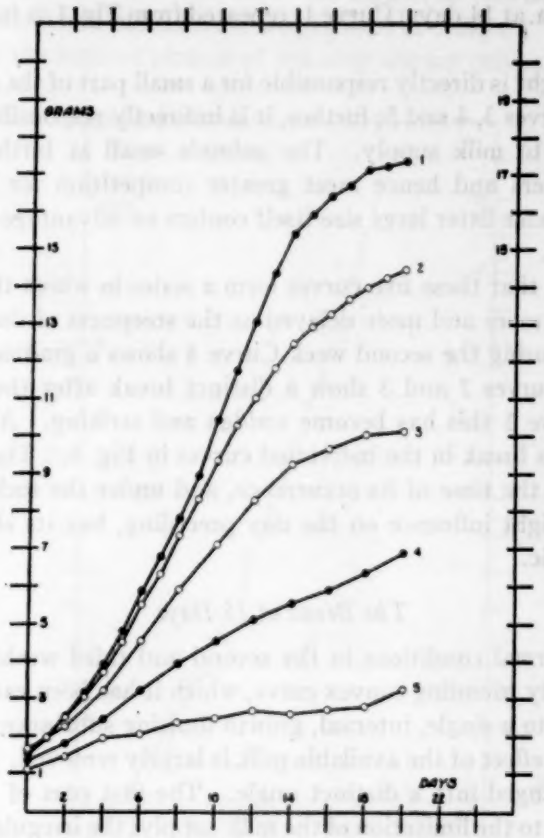

FIG. 6. Changes in the shape of the growth curve of the suckling mouse as the maximum is approached.

Curve 1: Maximum growth obtained; curve repeated from Fig. 1, Curve 1.

Curve 2: Averages for the six females in the present experiments nearest to 12.5 gm. at 14 days.

Curve 3: Averages for the six mice included in Curve 4, that were the heaviest at 14 days.

Curve 4: Averages of over 600 mice reported by Gates '25 (repeated from Fig. 1, Curve 10).

Curve 5: Averages of the six mice included in Curve 4, that were the lightest at 14 days.

six females from the current experiments that weighed between 12.25 and 12.50 gm. at 14 days; Curve 1, repeated from Fig. 1, is for the highest six.

Birth weight is directly responsible for a small part of the differences between Curves 3, 4 and 5; further, it is indirectly responsible through its relation to milk supply. The animals small at birth are born in large litters and hence meet greater competition for milk; and within the same litter large size itself confers an advantage in nursing competition.

It is clear that these five curves form a series in which the point of inflection is more and more delayed as the steepness of the curve increases. During the second week Curve 4 shows a gradual rounding off, while Curves 2 and 3 show a distinct break after the 14th day and in Curve 1 this has become sudden and striking. Attention is called to this break in the individual curves in Fig. 4. There is some variation in the time of its occurrence, and under the technique employed, a slight influence on the day preceding, but its sharpness is characteristic.

The Break at 15 Days

Under normal conditions in the second and third weeks, averages show a gently rounding convex curve, which it has been easy to interpret as due to a single, internal, growth limiting substance; but when the limiting effect of the available milk is largely removed, the smooth curve is changed into a distinct angle. The first part of the convex curve is due to the limitation of the milk supply; the irregularity of this limitation smooths the averages so that they grade imperceptibly into the second part which is due to some other limitation. New factors have suddenly become effective. Our observations indicate that this is a particularly significant and interesting moment in development. At this time comes a sudden change in behavior. Up to this time the large, well fed young are markedly inactive; the eyes have opened the day before; in another 24 hours they begin to run around; they pick up solid food and begin to nibble. (This agrees with the observations of Saller '27, p. 568.) They are indiscriminate in what they eat. If they have a chance they will eat the mother's feces; intestinal looseness develops. They can eat solid food, but are still dependent upon

their mothers, since the removal of the mother at this time will result in a continued and rapid loss of weight for 4 to 6 days. This is the initiation of the natural process of weaning; during this period they take

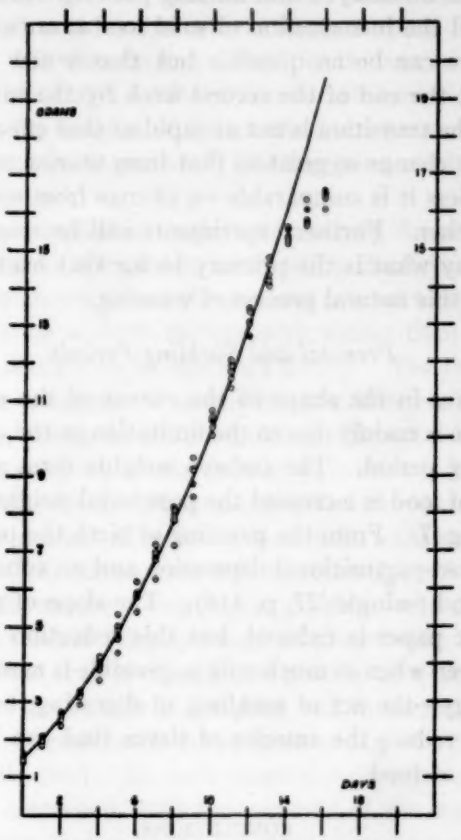

FIG. 7. Distributions of the individual weights of the six mice averaged in Fig. 1, Curve 1. Solid circles indicate two individuals with the same weight at the same age. The continuous line connects by straight lines the points for each day that lie along a parabola with the formula, $W = .0231T^{2.15}$, in which T equals the days from birth plus 7.

less mother's milk but not their full requirement of solid food. The completion of weaning is often indicated by an abrupt upward bend of the curve at a rate that is maintained. This may occur before the

mother is removed, but more often it is not until the mother is actually taken away that, after a slight loss for a day, the rapid phase is begun (see Fig. 2). By keeping all solid food away from the young, this rapid phase can be delayed and nursing prolonged; the depression is continued until the introduction of solid food at once starts the rapid growth. There can be no question but that a new phase of life is inaugurated at the end of the second week by the eating of the first solid food. The transition is not as rapid as that effected by parturition, nor is the change so great as that from uterine to free existence, but none the less it is comparable—a change from one source of nutrition to another. Further experiments will be required before attempting to say what is the primary factor that leads to this break, what initiates this natural process of weaning.

Prenatal and Suckling Periods

The difference in the shape of the curves of the embryo and the suckling mouse is mainly due to the limitation in the quantity of milk in the suckling period. The embryo weights form a parabola. As the quantity of food is increased the post-natal weights also approach a parabola, Fig. 7. From the morning of birth the parabola is taken up, with no post-parturitional depression and no subsequent rebound (Davenport and Swingle '27, p. 418). The slope of the straight line on logarithmic paper is reduced, but this reduction begins at birth. After birth, even when as much milk as possible is made available, new losses of energy—the act of suckling, of digesting, breathing, loss of surface heat—reduce the amount of tissue that can be made from a given amount of food.

CONCLUSIONS

Under usual conditions, the growth of the suckling mouse is limited by the quantity of mother's milk available.

As this limitation is removed the growth curve approaches a parabola, which is abruptly interrupted at the end of the second week, when the natural process of weaning begins.

DISCUSSION

The unreliability of mathematical analyses of growth curves as an approach to the fundamental growth processes, has been discussed by

Gray ('29). The present case would serve as further illustration of the importance of experimental analysis. Chemical methods are needed for the study of the chemistry of growth. The work of Needham ('25), Murray ('25, '26) and Cohn ('25) points the way. In contrast to analytical work of this type is the mathematical theorizing of Robertson ('26, '29) who, finding difficulties in describing mammalian growth in terms of a single autocatalytic chemical reaction, has evolved a theory calling for a series of independent master reactions coming into control at successive periods. The foregoing results do not favor this interpretation.

Robb ('29) has applied a single autocatalytic formula to the postnatal growth curve of the rabbit, interpreting the deviations from the theoretical curve as temporary depressions related to such changes in the conditions of life as birth and puberty, rather than to specific accelerations as called for by Robertson's theory. The present findings fully support the conclusion that the irregularities are in fact depressions related to a change in the conditions of life. In the mouse there appears to be no evidence of a necessary depression following parturition, but the relation of the break at the end of the second week to the beginning of the process of weaning is certain. Ostwald ('08) notes this; Robertson ('16) passes over this relationship, pointing out that the removal of the mother (weaning) at 21 days caused no distortion of the curve and hence no physiological disturbance in the young. We find that this is true if the natural process of weaning has been completed before the mother is removed, but in certain cases, especially good mothers continue to nurse their young and delay the complete shift to solid food. In these cases the depression in the curve of the young is continued until the removal of the mother. Saller, however, recognizes that weaning is a natural process that begins, as we find, on the 15th day. But he still agrees with Robertson that weaning does not cause the break in the curve because the break begins a week before the first eating of solid food. It is now clear that this break, usually found at the end of the first week, is not due to any deep seated feature of growth but to a purely external limitation which usually conceals the sudden nature of the break at 15 days. With the external limitations removed this break appears coincident with the change in food habits. It has been pointed out above that special

experiments are needed to determine the causal relationships between the events of this period. At present it is sufficient to recognize their correlation.

In the embryo trout, a declining food supply near the end of incubation is probably the cause of a break in the growth curve, which resembles the 15 day break in the mouse. Gray ('28) shows that by the time of this break (80 to 100 days) the yolk has become small and is rapidly growing smaller. The dependence of the size of the embryo on the amount of yolk is indicated by the facts that, (1) the growth rapidly rises as soon as extraneous food is eaten; (2) that large yolks produce large embryos; (3) that yolks experimentally reduced in size produce miniature embryos.

Besides showing this relationship between a period of slow growth and a change in the conditions of life, the present findings further support Robb's objection to the theory of growth "cycles" by making it clear that the slope of the curve immediately following weaning shows no acceleration when compared with that of the first 2 weeks when the food limitation is removed. Under normal conditions, the curve is steeper after weaning is completed, than before it begins.

On the application of a single continuous curve to the post-natal growth of the rabbit, certain questions arise. If the same curve fits the quiet nest life of the suckling period as well as the active post-weaning phase, should it not also fit the prenatal period? On the other hand, if growth during the suckling period in the rabbit is limited by the amount of mother's milk as it is in the mouse, it would be necessary to extend the interpretation of depression to cover this whole period. If the quantity of food is normally the limiting factor in the first part, and if the quantity of food after weaning is not limited, it may be necessary to reconsider the significance of a single mathematical curve fitting both periods. The continuity of the fit of a mathematical curve argues for its approach to the underlying processes of growth only so far as secondary influences do not vary. If the periods covered by the curve differ in endocrine development and function as well as in quantity and quality of food, the continuity of the curve may well argue against the theoretical interpretation drawn from the mathematical formula. The desirability of considering differ-

ent phases of life separately, as Schmalhausen has urged, seems manifest.

Taking different life phases separately, Schmalhausen ('29 with full references) has shown a surprising number of growth curves that approach a parabola. The significance of the parabola is fully discussed by Schmalhausen who considers that this inverse relationship between the velocity of growth and the length of time is the result of the regular reduction through differentiation of the relative amount of undifferentiated protoplasm which continues to grow exponentially. Thus the character of the growth curve is rather a phenomenon of differentiation than a measure of the nature of fundamental growth processes.

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SALT BRIDGES AND NEGATIVE VARIATIONS

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If a cell of *Nitella* imbibed with tap water be stimulated at such a point as *A* (Fig. 1) a negative variation usually passes to *B*, *D*, and *C*. If sufficient chloroform be applied at *D* the negative variation fails[1] to appear at *C* and we therefore speak of the chloroformed spot as a block.

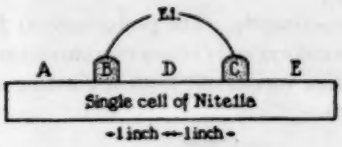

FIG. 1

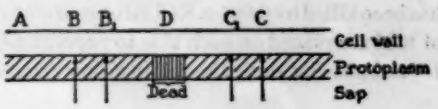

FIG. 2

FIG. 1. Diagram to show arrangement of experiments.

FIG. 2. Hypothetical diagram showing electrical conditions in the protoplasm. The arrows indicate E.M.F. (the direction shows how the positive current tends to flow). The protoplasm at *D* is shaded vertically to indicate that it has been killed by chloroform; it is assumed that it has been previously treated as in Fig. 4 to prevent negative variations from originating at *D*.

According to the local circuit theory[2] we should expect a variation starting at *A* to bring about a variation at *C* if we put a salt bridge around the chloroformed spot. For if the cell is imbibed with tap water (or 0.001 M KCl) we may diagram the protoplasm as in Fig. 2 and assume that a negative variation traveling from *A* to *B* makes an outward flow of current[3] at *B* causing[2c] the E.M.F. at *B* to approach zero and a flow

[1] It is necessary to arrange the experiments so that no negative variation originates at *D*. See footnote 2 *c* and Fig. 4.

[2] *Cf.* (*a*) Lillie, R. S., Protoplasmic action and nervous action, University of Chicago Press, Chicago, 1923; (*b*) Davis, H., *Physiol. Reviews*, 1926, **6**, 547; (*c*) Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 391.

[3] By this is meant the sort of flow indicated by the arrow at B_1 in Fig. 3.

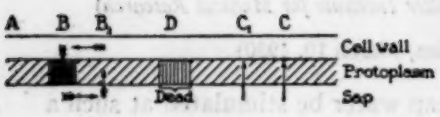

FIG. 3

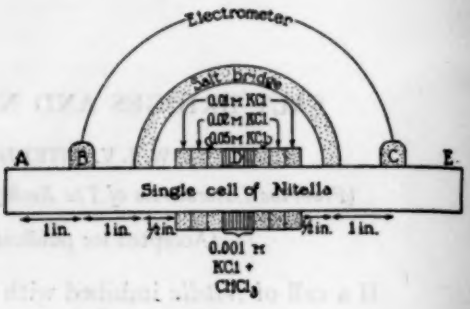

FIG. 4

FIG. 3. As in Fig. 2 but showing a flow of current between B and B_1 (flow is indicated by feathered arrows, E.M.F. by plain arrows). The protoplasm at B is shaded horizontally to indicate that it has temporarily lost its potential (that at D is shaded vertically to indicate that it is dead).

FIG. 4. Diagram to show arrangement of experiments. The protoplasm at D has been killed by 0.001 M KCl saturated with chloroform and various concentrations of KCl are placed on each side to prevent negative variations[2e] from originating at D (and passing along the cell in either direction).

FIG. 5

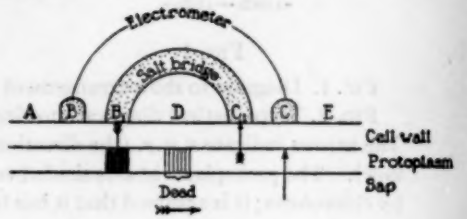

FIG. 6

FIG. 5. As in Fig. 4 but without a salt bridge: the protoplasm at B_1 has lost its potential but the P.D. gradient is not steep enough to cause sufficient outward flow at C_1 to start a variation. The arrows indicate E.M.F.

FIG. 6. As in Fig. 5 but with a flow of current through a salt bridge as indicated by the feathered arrows (the horizontal flow in the protoplasm and cell wall is relatively small and is therefore not indicated). Plain arrows indicate E.M.F. and feathered arrows indicate current.

to start between B and B_1 as shown in Fig. 3. This in turn will cause the E.M.F. at B_1 to approach zero and in this way the variation will travel along the cell.[4]

[4] *Cf.* Blinks, L. R., Harris, E. S., and Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 836.

The situation when a block is placed in the center (as in Fig. 4 but without the salt bridge) probably resembles that shown in Fig. 5. The P.D. gradient is not steep enough to permit sufficient outward flow at C_1 to reduce its E.M.F. to zero and start a negative variation.

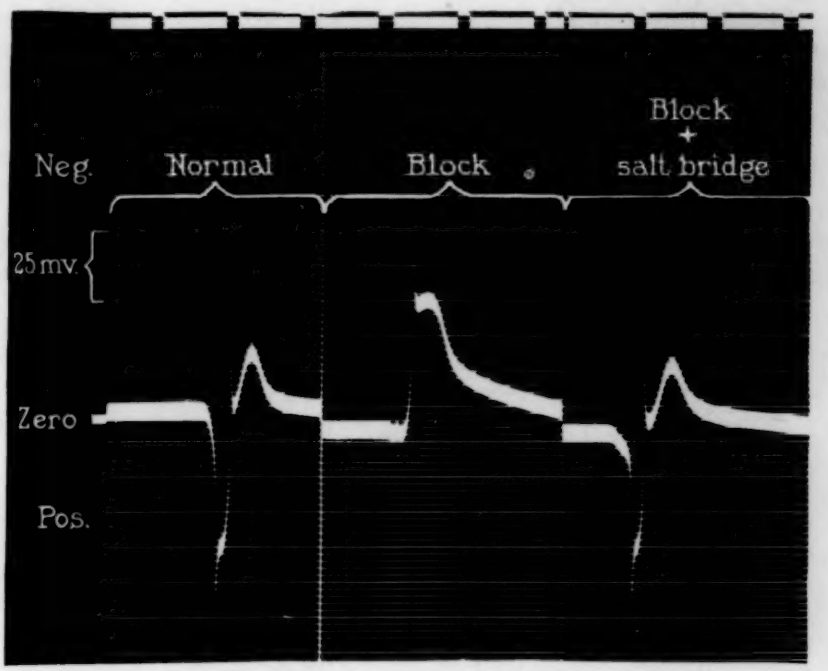

FIG. 7. Photographic record showing the P.D. of *B* in relation to *C*.

The part marked "normal" shows a diphasic action current, the experiment being arranged as in Fig. 1 (with 0.001 M KCl at *B* and *C*); the cell is stimulated at *E* (by applying 0.05 M KCl^{2c}) and the negative variation reaching *C* makes *B* appear positive because we are recording the P.D. of *B* with reference to *C*; afterward it reaches *B* making it appear negative.

The part marked "block" shows the effect of stimulation at *A* when the experiment is arranged as in Fig. 4 but without a salt bridge. A negative variation starting at *A* reaches *B* but since it cannot pass *D* the action current is monophasic.

The part marked "block plus salt bridge" shows the action current produced when the experiment is arranged as in Fig. 4 (with salt bridge). The action current produced by stimulation at *E* is diphasic like the one labelled "normal" (at the start of the record) for reasons given in the text.

The time marks represent 5-second intervals.

But if we put a salt bridge[5] between B_1 and C_1 and the E.M.F. at B_1 falls to zero (as the result of a negative variation) a flow can take place as shown in Fig. 6, which will reduce the E.M.F. at C_1 to zero, starting a negative variation.[6] We thus obtain the record shown in Fig. 7.

The experiment referred to in Fig. 7 was arranged as in Fig. 4, putting a piece of cotton soaked in 0.001 M KCl saturated with chloroform in the middle of the cell

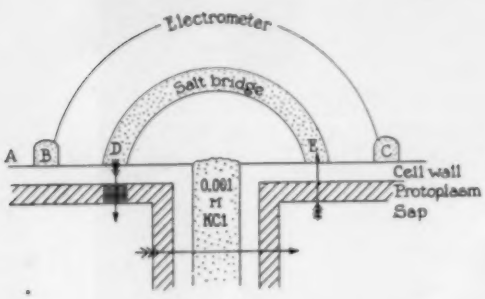

FIG. 8

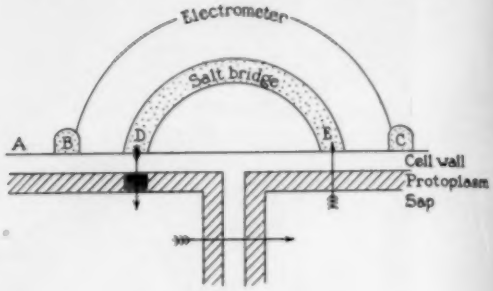

FIG. 9

FIG. 8. Hypothetical diagram of electrical conditions in two cells (from different plants) in contact with cotton soaked with 0.001 M KCl and connected by a salt bridge. The protoplasm at D has lost its potential (as indicated by the horizontal shading) and in consequence there is a flow of current between D and E as indicated by the feathered arrows. (Since the horizontal flow in the horizontal cell walls and the vertical flow in the vertical cell walls is relatively small it is not indicated.)

FIG. 9. Hypothetical diagram of electrical conditions in two cells in their natural union: they are connected by a salt bridge. The protoplasm at D has lost its potential as indicated by the horizontal shading and in consequence there is a flow of current as indicated by the feathered arrows (since the horizontal flow in the horizontal cell walls and the vertical flow in the vertical cell walls is relatively small it is not indicated).

and on each side pieces of cotton soaked in various concentrations of KCl (as shown in the figure) so arranged as to make a gradual gradient (this, as shown in a previous paper, prevents the chloroformed spot from starting negative variations). At B and C 0.001 M KCl was applied and 0.05 M KCl was placed at A or E to start a series of negative variations.

The salt bridge was composed of cotton soaked in 0.001 M KCl in all cases.

All experiments were performed on *Nitella flexilis* at 19° to 20°C. the technique being that described in previous papers unless otherwise stated.

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[5] Two calomel electrodes connected by a wire may serve.

[6] This would, of course, travel in both directions.

In view of this result it does not seem strange that a salt bridge enables a negative variation to set up a similar variation even in a cell taken from another plant and placed beside it at a distance of half an inch or more, as shown[7] in Fig. 8. In this case the circuit passes 4 times through living protoplasm (instead of twice, as in the preceding experiments). Since the resistance of the protoplasm is very high[8] we might expect the flow of current to be greatly reduced but in spite of this a negative variation starting at *A* and reaching *D* is promptly followed by one in the other cell when the salt bridge has a sufficiently low resistance (*e.g.*, cotton soaked in 0.001 M KCl).

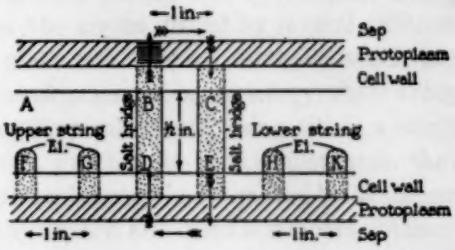

FIG. 10. Hypothetical diagram of electrical conditions in two cells (from different plants) connected by two salt bridges. The protoplasm at *B* has lost its potential (as indicated by the horizontal shading) and in consequence there is a flow of current as indicated by the feathered arrows. Since the horizontal flow in the protoplasm and in the horizontal cell walls is relatively small it is not indicated.

If an experiment be arranged as in Fig. 9, employing two cells in their natural union, the negative variation starting at *A* and reaching *D* can start a variation at *E* by the aid of a salt bridge but this happens much less frequently when the salt bridge is absent: apparently the cell wall may act as a salt bridge in some cases, as was observed by Mr. Harris and previously reported.[4]

A positive result is also obtained when the experiment is arranged as in Fig. 10. In this case the currents would presumably be as indi-

[7] Experiments arranged as in Figs. 8 and 9 were checked in all cases by using a double string galvanometer and employing two contacts connected with one string on one side of the salt bridge and on the other side two contacts connected to the other string as in Fig. 10.

[8] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, 13, 495.

cated so that in the lower cell there would be an outward current at *D* which could stimulate. This is commonly the case. Preliminary experiments of this sort were carried out by Mr. Harris and have been previously reported.[4]

SUMMARY

A negative variation in *Nitella* is unable to pass a spot killed by chloroform but can set up a negative variation beyond this spot when a salt bridge is put around it. It can likewise set up a negative variation in a cell of another plant if connected to it by two salt bridges.

A METHOD FOR THE QUANTITATIVE ESTIMATION OF BACTERIA IN SUSPENSIONS

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(Accepted for publication, March 19, 1930)

Determinations of the number of bacterial cells present in a given suspension may be accomplished by several different means. Besides the classical plate and dilution methods which have contributed so much to the development of bacteriology, there are suitable procedures for direct microscopical counts, using either a counting chamber or a stained mount. Further, in certain instances, the total cell concentration of a suspension may be estimated by nephelometric comparison, by weighing the washed and dried sediments obtained from centrifuged aliquots, or by measuring a product of metabolism.

For some types of work the volume occupied by organisms has proven a satisfactory measure of the number of cells. Total cell volume is determined by centrifuging, the sediment being deposited in a calibrated cup from which the volume of packed cells is read directly. Having already established the volume-cell number ratio, preferably by direct count, volume readings are readily convertible into total cells per cubic centimeter.

Paine (1), Carlson (2), and Slator (3) have reported favorable experiences with such a technic used by each of them to determine the cell content of yeast cultures, while Hopkins (4) has successfully utilized the method for standardizing bacterial vaccines.

During the course of a study of bacteriophage action on staphylococci there arose a two-fold necessity: first, for a method by which estimates of cell numbers in bacteriophage-containing suspensions might be obtained; and second, for a technic giving rapid and accurate cell number determinations in dense preparations to be used for making up dilutions of known bacterial concentrations. Obviously, the plate

and dilution methods could not be employed in the presence of bacteriophage, and neither procedure would furnish immediate information for preparing cell dilutions. It was finally found possible to adapt the centrifuged-sediment method to these problems with satisfactory results.

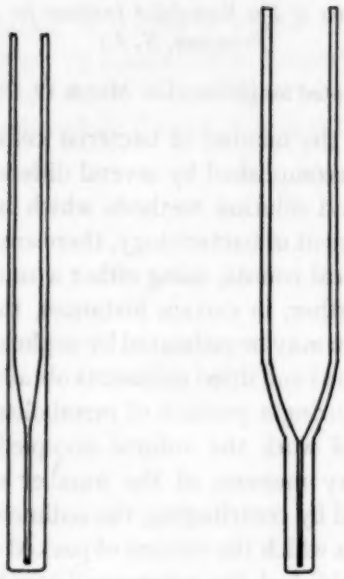

FIG. 1 FIG. 2

FIG. 1. Small centrifuge tube for dense suspensions. Length: 11 cm. Outside diameter: 8 mm. Length of capillary: 25 mm. Capillary bore: 1 mm. Small drop of mercury at bottom to give clear meniscus.

FIG. 2. Large centrifuge tube for light suspensions. Length: 13 cm. Outside diameter: 1.7 cm. Length of capillary: 30 mm. Capillary bore: 0.6 mm. Small drop of mercury at bottom to give clear meniscus.

The centrifuge tubes (Fig. 1) constitute the essential feature of the method. They are calibrated individually with dense cell suspensions which are made up in buffer solution, and standardized by direct count. Five consecutive 1 ml. aliquots are centrifuged at known speed for a period of time sufficient to insure maximal packing. The column length of the sediment is measured in each instance with a

cathetometer or vernier calipers and the average bacterial content per millimeter of tube length calculated. Both menisci are level and sharp and permit accurate measurement.

Tubes calibrated in this way were employed in actual routine as follows: 16 hour cultures of a single strain of *Staphylococcus aureus* grown in Blake flasks were washed from the agar with $\frac{M}{200}$ phosphate buffer of pH 7.6 using 30 ml. to each flask. The emulsion was filtered through a sterile Schleicher and Schüll Faltenfilter No. 588 and 1 ml. aliquots centrifuged in the small tubes (Fig. 1) at 2600 R.P.M. for 15 minutes. The lengths of the columns of packed bacterial cells were then measured and the figures converted into cells per milliliter of original suspension. From such suspensions dilutions of any required cell content were of course readily prepared.

The difference between consecutive measurements of the same suspension was found to be <1.0 per cent (triplicate determinations on 65 emulsions), while the total error based on repeated checks with careful direct counts was consistently <2 per cent. These figures apply to the measurement of very dense suspensions containing approximately 14×10^9 cells/ml. and giving a column length of about 20 mm.

In the case of light suspensions of staphylococci in nutrient broth, 10 ml. aliquots added to 2 ml. of a mixture of acetone 10 ml. $\frac{M}{1}$ NaCl 3.0 ml., neutral formalin 2.0 ml., were centrifuged at 2600 R.P.M. for 15 minutes in large cups (Fig. 2). These cups were calibrated with light suspensions of known cell content (direct count), which were mixed with the acetone-salt-formalin reagent before centrifuging. Here a larger percentage of the organisms remained in the supernatant fluid and the difference between successive determinations on the same suspension was greater than in the case of the dense preparations, averaging approximately 4 per cent. The total error based on direct count figures averaged 5 per cent.

SUMMARY

A method is described for estimating the cell concentration of bacterial suspensions by measurement of the column lengths of packed

cells centrifuged into a capillary tube of fine bore. The total error, under the experimental conditions employed, checked by direct count, was <2 per cent for dense suspensions and about 5 per cent for light suspensions. The method is suitable for work requiring rapid and accurate routine preparation of bacterial suspensions of known cell concentrations.

The writer wishes to express his indebtedness to Dr. John H. Northrop for generous aid and guidance and to Miss Virginia Toussaint for faithful technical assistance.

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A METHOD FOR THE QUANTITATIVE DETERMINATION OF BACTERIOPHAGE

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Two general methods for the quantitative determination of bacteriophage are known. The first of these involves testing serial dilutions of the lytic agent for ability to produce complete dissolution of a broth culture of susceptible bacteria. The last effective dilution is divided into several aliquots which are in turn seeded with phage-susceptible organisms. Thus if four 1 ml. portions of a 10^{-6} dilution of a phage suspension produce lysis and six do not, it is assumed that each of the four active samples contains at least 1 lytic particle and that therefore the original lysate possesses a minimum concentration of 4×10^6 phage corpuscles/ml.

The alternate method consists of plating appropriate phage dilutions upon a substrate of susceptible bacteria. The round, punched-out lytic areas are considered to represent the loci of single phage particles, and a count of their numbers should furnish a simple measure of the total phage per milliliter.

D'Herelle has fully described and discussed both procedures and apparently has been able to obtain satisfactory checks with them. In judging the suitability of these technics for critical quantitative work, however, it would appear that even in d'Herelle's hands agreement of results obtained by the two methods has been wanting. To quote d'Herelle (1) "The undiluted suspension . . . . of the more powerful race contained 200,000 million (2×10^{11}) corpuscles per cc. according to the dilution method and 121,000 (1.21×10^{11}) according to the plaque method. For the second race the number of corpuscles was 6×10^9 by the first method and 8.1×10^9 by the second." The extreme possible errors in the cited cases are 65 per cent and 35 per cent respectively.

Clark (2) has analyzed the dilution method of titration from the statistical viewpoint and concludes that with a dilution constant of 0.1 only about 60 per cent of parallel runs on the same solution should give the same end-point. It is

apparent that a difference of 1 tube in the end-point of a series of this sort is apt to produce a considerable error in the final calculation. A recent survey by the writer of previous titration data of his own has given a figure but little greater than the 60 per cent of Clark's.

Plaque counts appear to offer no particular advantage over the dilution technic as far as agreement between parallel runs is concerned. Several factors other than the concentration of phage have been shown to influence markedly the number of plaques appearing on solid media (3) and considerable personal experience with the method has led the writer to the conclusion that, in his hands at least, it is inadequate for accurate determinations.

In attempting to devise some more suitable technic the reaction between a highly active antistaphylococcus phage and a single strain of *S. aureus* was studied. Viscosimetric measurements of bacterial swelling following the addition of phage to dense suspensions failed to furnish any basis for quantitative phage estimations. Determinations of the number of bacteria required to completely adsorb phage from exposed lysates resulted similarly.

As a next step a study of the effects produced by varying the C_{Phage} and $C_{\text{Bact.}}$ present in a given volume at the beginning of the reaction was undertaken. Although it is stated (4) that "within certain very wide limits the quantity of bacteriophage filtrate necessary to inoculate to cause this dissolution is a matter of no moment; the course of the action and the final result of the phenomenon is the same, whether the amount inoculated is 1 cc. or 0.001, to 10 cc. of medium," this was not found to be the case in the present instance.

Marked differences in the course of the reaction were found to occur upon varying the concentrations of the reactants. For example, under the experimental conditions described below, 4 ml. of 1 per cent phage added to 1 ml. of a broth suspension of staphylococci containing a total of 12.5×10^7 cells produced clearing in 1.9 hours, whereas a similar preparation made with 0.01 per cent phage required 3 hours for the completion of lysis. Comparable results over a wide range of phage concentrations in a constant total volume of 5 ml. containing 12.5×10^7 cells suggested that under these conditions lysis is a function of C_{Phage} and time, and that quantitative estimations of phage could be made either by determining the length of time required to produce clearing of a culture containing a certain number of bacteria at the beginning of the process or by determining the number of bacteria

lysed in a given time. Upon this general basis the following procedure was developed.

Details of Method for Quantitative Phage Determination as Used in Present Work with S. aureus and Antistaphylococcus Phage

A considerable quantity of phage is prepared by adding bacteriophage and susceptible organisms to nutrient broth of pH 7.6 in such amounts that respective concentrations of 1.0 per cent phage and 2.0×10^7 cells/ml. are obtained. The mixed reactants are rocked at 36°C. for 3 hours by which time lysis is complete. The lysate is stored in liter flasks at 5°C. *without filtering*. Under such conditions it has been found to maintain full strength satisfactorily over a period of months.

Units: Bacteriophage, like enzymes, can only be measured by its activity and the units employed therefore will have the dimensions of a velocity constant; *i.e.*

TABLE I

Amounts of S. aureus and Antistaphylococcus Phage Used in Quantitative Phage Determinations

$C_{Bact.}$ in suspension	Amount of bacterial suspension used	C_{Phage} used		Amount of phage dilution used	$C_{Bact.}$ in mixture
		per cent	*units*		
12.5×10^7/ml.	1 ml.	0.01	1×10^8	4 ml.	2.5×10^7/ml.
"	"	.001	1×10^6	"	"
"	"	.0001	1×10^4	"	"

in this case the number of bacteria lysed per unit of time per unit of phage. The rational unit would be that quantity which would lyse one bacterium in one second but since this cannot be determined experimentally it is necessary to choose an arbitrary number. The rate of lysis varies with temperature, pH, and condition of the culture; consequently these variables must also be fixed. The unit of phage used in this paper is defined as the minimum quantity which will cause complete clearing of 1.25×10^8 cells of *S. aureus* (prepared from a 16 hour culture grown on agar) in 5 ml. pH 7.6 broth at 36°C. 1×10^{-10} ml. of the "standard" phage suspension prepared as described above suffices to produce lysis under these conditions and therefore contains 1×10^{10} units per ml. On this basis a 1 per cent phage suspension will contain 1×10^8 units/ml., a 0.01 per cent suspension 1×10^6 units/ml., etc. The working of this scale in practise is brought out below.

A 16 hour culture of the test organism grown in Blake flasks is washed from the surface of the agar with saline solution. The resulting emulsion is filtered through a Schleicher and Schüll Faltenfilter No. 588 and its bacterial content determined

by the centrifuged-sediment method (5). Dilutions in broth are immediately prepared and thoroughly mixed with standard phage dilutions in the amounts shown in Table I. Tubes of uniform diameter and any convenient size are employed. We regularly use large tubes of 15 mm. inside diameter because of the accuracy with which they may be read. They are placed in a mechanical shaker mounted in a water bath maintained at 36°C. and are read at short intervals as described below.

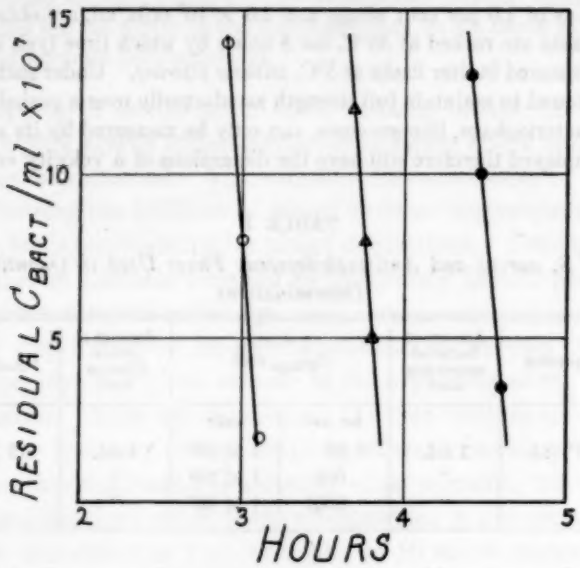

FIG. 1. Residual bacterial concentrations during lysis by various amounts of phage plotted against time

○ 1×10^6 Units Phage
△ 1×10^5 " "
● 1×10^4 " "

Readings are made by comparing the turbidity of the lysing suspension with formalinized bacterial suspensions of known cell concentrations contained in standard tubes. A series of 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 20 $\times 10^7$ staphylococci/ml. in broth containing 5 per cent formalin is prepared each day for this purpose.

The light source for reading is important. A satisfactory arrangement is made by mounting a bullet-shaped daylight microscope lamp just below the level of the eyes, turning it at an angle of 45° with the vertical. The tubes are held in front of and slightly below the daylight filter so that they are illuminated against a dark background. Considerable Tyndall effect is obtained in this way and suspensions

of 9 × 10^7 and 10 × 10^7 staphylococci/ml., for example, may be readily differentiated.

Growth of bacteria occurs in all the tubes and turbidity increases greatly. Readings are begun when the first diminution in turbidity becomes apparent and are recorded graphically by plotting the residual bacterial concentrations as ordinates against the time required to reach these values. Curves of the type indicated in Fig. 1 are obtained. On such curves one chooses a convenient end-point (here 8 × 10^7 Bact./ml.) and interpolates a second curve, Fig. 2, by plotting the logs of the phage units required to bring the original bacterial suspension to this arbi-

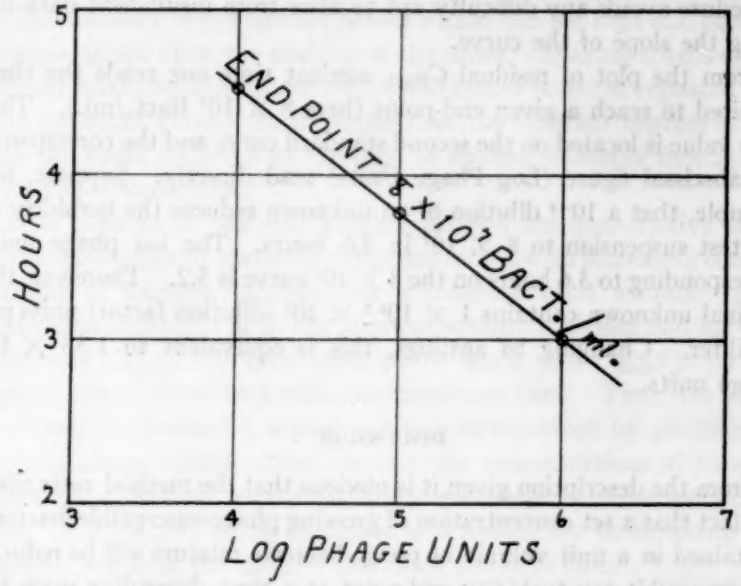

FIG. 2. Log of C_{Phage} (units) plotted against the time required to reduce $C_{Bact.}$ to 8 × 10^7/ml.

trary end-point against the time spent in the process. Both sets of curves are reproducible and may be quite closely fixed. However, in titrating unknowns it is best to include two known phage dilutions in each set-up. Readings from the control tubes may shift the second curve slightly but will not alter the slope.

An unknown is run as follows. 4 ml. aliquots of the undiluted lysate and four 1:10 serial dilutions in broth are pipetted into standard tubes. To each is added 1 ml. of a broth suspension of staphylococci prepared as described above and containing 12.5 × 10^7 Bact./ml. The tubes are mounted in the water bath shaker and at 2.5 hours readings are

begun. Any original C_{Phage} between 100 per cent (1×10^{10} units) and 0.0001 per cent (1×10^{4} units) will give in at least one of the five tubes a sharp decrement in $C_{Bact.}$ at some time between 3 and 4.5 hours. It is essential to catch this explosive process of lysis so that two or preferably three points may be established for drawing the curve of Fig. 1. In order to be sure of this the tubes are read every 0.2 hour between 2.5 and 4.7 hours. When the turbidity in any tube drops below 20×10^{7} Bact./ml. that tube is read every 0.1 hour. Such a procedure avoids any difficulty apt to arise from insufficient data for fixing the slope of the curve.

From the plot of residual $C_{Bact.}$ against time one reads the time required to reach a given end-point (here 8×10^{7} Bact./ml.). This time value is located on the second standard curve and the corresponding abscissal figure (Log Phage Units) read directly. Suppose, for example, that a 10^{-3} dilution of an unknown reduces the turbidity of the test suspension to 8×10^{7} in 3.6 hours. The log phage units corresponding to 3.6 hours on the 8×10^{7} curve is 5.2. Therefore, the original unknown contains $1 \times 10^{5.2} \times 10^{3}$ (dilution factor) units per milliliter. Changing to antilogs, this is equivalent to 1.58×10^{8} phage units.

DISCUSSION

From the description given it is obvious that the method rests upon the fact that a set concentration of growing phage-susceptible bacteria contained in a unit volume of phage-bacteria mixture will be reduced to some arbitrary turbidity end-point at a time depending upon the number of phage units present at the start of the process. The amounts of phage involved in the reaction are conveniently expressed by defining the standard phage as containing 1×10^{10} units. It should be stated that this figure is not the actual number of lytic particles in the standard; it is a purely arbitrary constant adopted for convenience. In the actual test 4 ml. of phage or phage dilution are used while titer calculations are based upon 1 ml. quantities. No correction for this difference need be made since the 4 ml. quantity is constant throughout.

The original $C_{Bact.}$, the turbidity end-point, the total volume, and the C_{Phage} range used for estimating unknowns, are again not set

values but are fixed in each instance by preliminary runs with the particular phage and bacterium being studied.

In the present case phage values $<1 \times 10^4$ units/ml. did not give points lying on the curve of Fig. 2. Such irregularity is to be expected in very high dilutions and the range for quantitative estimations must be chosen with this in mind. Similarly, concentrated lysates will not fit the curve because the turbidity of the suspension never develops sufficiently to furnish the required end-point.

The accuracy of the method is well within ±5 per cent and depends to some extent upon the shaking of the tubes. Shaking speeds up the process markedly and decreases the differences noted in repeated runs with unshaken tubes. In the latter case the different degrees of jarring the tubes are given at the time of reading probably account for such minor variations.

It is possible to use still another method for quantitative work. Various dilutions of standard phage are mixed with increasing concentrations of bacteria, maintaining a constant total volume. Some convenient end-point as in the above procedure is chosen and for each particular C_{Phage} the time required to reach this $C_{\text{Bact.}}$ is plotted against the original bacterial concentrations used. From the series of curves so obtained a second curve is interpolated by plotting the original phage values (units) against the concentrations of bacteria which they reduced to the turbidity end-point at some particular time. The second curve furnishes a standard for determining unknowns just as in the method described above in detail.

CONCLUSIONS

1. In the case of the staphylococcus and antistaphylococcus phage studied, with the total volume of the mixture being kept constant, there exists a definite quantitative relationship between C_{Phage} and the time required to reduce a particular concentration of growing phage-susceptible bacteria to an arbitrary turbidity end-point.

2. This relationship furnishes a basis for the quantitative estimation of bacteriophage. A method is described having an accuracy within ±5 per cent.

It is a pleasure to acknowledge the aid and interest of Dr. John H. Northrop in this work and the faithful technical assistance of Miss V. Toussaint.

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ELASTICITY, DOUBLE REFRACTION AND SWELLING OF ISOELECTRIC GELATIN

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(Accepted for publication, March 19, 1930)

The importance of the rôle which elasticity plays in the mechanism of swelling of a gelatin gel is well recognized in Procter-Wilson's theory of swelling of gelatin in acid or alkali. According to this theory (1) there are two opposing forces acting on a block of gel when placed in an aqueous solution of acid or alkali, first, the osmotic force which is brought about by the excess of diffusible ions in the gel over those in the outside solution and which causes the gel to swell, and second, the elastic force produced by the rigidity of the gel structure and which limits the extent of swelling. At equilibrium the two forces balance each other. Thus the elasticity of the gel plays the same rôle in the swelling of gelatin as hydrostatic pressure in osmosis phenomena.

Procter-Wilson's theory of swelling has been applied by Northrop and the writer (2) to the mechanism of swelling of isoelectric gelatin in the absence of acid or alkali. Swelling in this case is caused by the presence in gelatin of a soluble fraction which produces osmotic pressure. But the swelling in this case also is regulated by the elasticity of the block as a whole. When a gelatin solution is cooled and allowed to set, the gel formed is under no elastic stress, but as soon as it is placed in water and allowed to swell the gel undergoes an elastic strain producing a stress in it which resists the swelling until an equilibrium is established between the two opposing forces.

Both the equilibrium conditions of swelling of isoelectric gelatin (3) as well as the kinetics of swelling (4) have been found to agree quantitatively with the described theory. The peculiar phenomena observed by Arisz (5) and others that gels of various concentrations when dried to an identical high concentration swell rapidly to their original concentrations and then take up water slowly, and also the effect of

varying temperatures, were readily explained by Northrop (3) on the basis that the gel at setting is under no elastic stress, but on drying the gels are put under a compressive stress which makes the gels swell rapidly to their original volume when placed in water. A block of gelatin which has swollen to a constant value at a low temperature and has then been removed from the water and kept for about an hour at a higher temperature, continues to swell when replaced in cold water. The raising of temperature destroys the internal stresses in the gel, and allows the gel to swell more under the influence of osmotic forces.

It is thus evident that a knowledge of the elastic properties of gels is as important as that of the osmotic pressure phenomena to a clear understanding of the mechanism of swelling.

A great deal of work has been done on the determination of the elastic constants of gels.[1] In practically all of these studies the observations were made on the elastic properties of gels during mechanical deformation such as stretching, compressing or twisting.

But, as stated above, elastic stresses may be induced in a gel by simply allowing it to swell in cold water. Hence attempts were made in a previous work (6) by Northrop and the writer to determine the effect of salts on the bulk modulus of elasticity of gelatin during swelling from data on osmotic pressure and swelling of gelatin in the presence of salts, under the assumption that the osmotic pressure of gelatin solutions is proportional to the swelling pressure.

A method was developed (7) to measure directly the swelling pressure of gels of various gelatin contents. At equilibrium this equals the internal elastic stress. Calculations were thus made of the bulk modulus of elasticity of gels of various concentrations of gelatin at swelling; but this method is quite complicated and subject to many errors.

[1] A long list of old references on this subject is given by H. Freundlich in his Kapillarchemie, Leipzig, 3rd edition, 1923, 986; and also by Harry B. Weiser in his article on "Jellies and gelatinous precipitates" in Bogue, R. H., The theory and application of colloidal behavior, New York, 1924, **1**, 402. Important studies of the elastic properties of gels have been made recently in this country by S. E. Sheppard and his collaborators, *J. Am. Chem. Soc.*, 1921, **43**, 539; 1922, **44**, 1857; *J. Ind. and Eng. Chem.*, 1923, **15**, 571; 1924, **16**, 593; and also by G. W. Scarth, *J. Phys. Chem.*, 1925, **29**, 1009.

A more direct and simple method, which has been employed in this work, is to determine internal elastic stresses by measuring the double refraction produced in the gel during swelling. This method of measuring internal stresses is employed quite extensively in engineering as a means of studying internal stresses in cases which are difficult to analyze mathematically. "Thus Coker (9) was able by means of loaded transparent zylonite models to verify well-known calculations on stress distribution such as those for hook sections and perforated tie bars, besides very numerous cases of material having discontinuities such as notches of various kinds, where exact mathematical treatment is practically impossible."[2]

Double Refraction in Materials Subjected to Mechanical Stresses

It is not the intention of the writer to enter here into a general discussion of double refraction, a subject which is fully discussed in books on optics and optical crystallography.[3] The few remarks given here have the object only to define the terms and symbols used in the experimental part of this work.

It is known since Brewster's studies (Philos. Trans., 1816) that when a strip of an isotropic material such as glass, resin, or gelatin, is put under a mechanical stress like tension or compression it becomes optically double refractive.

This is observed by placing the strip of material on the stage of a polarizing microscope between crossed Nicol prisms and exerting the tension or compression in a direction of 45° with the directions of the vibration planes of the crossed Nicols. The plane polarized beam of light from the polarizer or lower Nicol is resolved on entering the strained material into two component beams, one of which has its vibrations in a plane parallel to the direction of the applied stress while the other beam has its vibration in a plane perpendicular to it. There is also a difference in the velocity with which these component beams of light travel through the strained material. In the case of most materials the faster beam is in the direction of tension, while if

[2] Cited from Morley, A., Strength of materials, London, 1920.

[3] An excellent and clear discussion of the whole subject as applied to colloids is given in Ambronn-Frey's little book, Das polarisationsmikroskop, Leipzig, 1926.

the material is compressed the beam of light whose plane of vibration is in the direction of compression is the slower one.

On entering the upper Nicol the two beams of light are made to travel along the same path after they have traveled with a different velocity through the doubly refractive medium, and interference results.

Let V_0 be the velocity of the beam of light in air, which is about the same as in a vacuum, and V_1 and V_2 the velocities of the component beams in the doubly refractive medium, V_1 being the velocity of the faster beam. Let also λ_0, λ_1, and λ_2 be the corresponding wave lengths of the beams. Since the color of the light is not affected by passing through the strained colorless material it follows that f, the frequency of vibration, which is the factor that determines color, is the same for the component beams as for the original one.

Hence

$$\lambda_0 = \frac{V_0}{f}$$

$$\lambda_1 = \frac{V_1}{f}$$

$$\lambda_2 = \frac{V_2}{f}$$

or

$$\lambda_1 = \lambda_0 \frac{V_1}{V_0} = \frac{\lambda_0}{n_1}$$

and

$$\lambda_2 = \lambda_0 \frac{V_2}{V_0} = \frac{\lambda_0}{n_2}$$

where n_1 and n_2 are the indices of refraction of the material in the direction of the stress and in the direction perpendicular to it.

The faster beam of light in passing through a distance, s, of the strained material will complete $\frac{s}{\lambda_1} = \frac{s\, n_1}{\lambda_0}$ wave lengths, or vibrations, while the slower beam will complete a greater number of wave lengths in passing through the same distance, s,

namely

$$\frac{s}{\lambda_2} = \frac{sn_2}{\lambda_0}$$

Since the time required for one vibration is the same for both beams it follows that the slower beam will be retarded by the time required to complete

$$\frac{sn_2}{\lambda_0} - \frac{sn_1}{\lambda_0} = \frac{s}{\lambda_0}(n_2 - n_1) = \Delta p \qquad \text{(Equation 1)}$$

wave lengths. This retardation or phase difference which is expressed in wave lengths or fractions of a wave length is the cause of the interference colors observed, with white light as a source of illumination, when the two beams are made to pass through the same path through the upper Nicol. The interference colors thus produced serve as an approximate measure of the phase difference between the two beams of polarized light.

The magnitude of double refraction is defined by the value of $n_2 - n_1$, *i.e.*, by the difference between the two indices of refraction. There are various methods for measuring accurately the phase difference in double refraction. The most accurate and convenient to use is the Babinet compensator method, a detailed description of which is given by Johannsen (9).

If light of a known wave length λ_0 is employed then the value of $n_2 - n_1$ is readily determined.

The Relation between Double Refraction and Elastic Strain

The theory of the relation between double refraction and elastic strain in an isotropic body was developed by Neumann (10). He considered the case of a body which is under strain in three dimensions, *i.e.*, under a volume strain. Let a, b, and c be the three dimensions of an isotropic body, and let α, β, and γ, be the strains in the three directions, *i.e.*,

$$\alpha = \frac{\Delta a}{a}, \quad \beta = \frac{\Delta b}{b}, \quad \text{and } \gamma = \frac{\Delta c}{c}$$

then, according to Neumann, the velocity, V_1 of light in the isotropic medium when under no strain is changed to

$$A = V_1 + q\alpha + p\beta + p\gamma$$
$$B = V_1 + p\alpha + q\beta + p\gamma$$
$$C = V_1 + p\alpha + p\beta + q\gamma$$

where A, B, and C are the velocities of light in directions of a, b, and c, and q and p are constants depending on the material.

The technique of measuring double refraction determines the differences between the indices of refraction in the medium in directions lying in a plane perpendicular to the direction of observation. If the observation (*i.e.*, the axis of the polariscope) is in the direction of c, then the observed phase difference, Δ_p, is proportional to the difference between the indices of refraction in the directions of a, and b, *i.e.*,

$$\Delta p = \frac{c}{\lambda_0}\left(\frac{V_0}{A} - \frac{V_0}{B}\right)$$

But

$$\frac{V_0}{A} - \frac{V_0}{B} = \frac{V_0(V_1 + p\alpha + q\beta + p\gamma - V_1 - q\alpha - p\beta - p\gamma)}{A \times B}$$

or

$$\frac{V_0}{A} - \frac{V_0}{B} = \frac{V_0(p - q)(\alpha - \beta)}{A \times B}$$

Since $V_1 - A$ and $V_1 - B$ are very small numbers as compared with the values of A, B and V_1, therefore

$$\frac{V_0}{A} - \frac{V_0}{B} = \frac{V_0(p - q)(\alpha - \beta)}{V_1^2}$$

$\frac{V_0}{V_1^2}$ as well as $p - q$ are constant for a given material and a given monochromatic source of light. It follows then that when a body is under a bulk strain the observed double refraction is proportional to the difference in the linear strains in the directions lying in a plane perpendicular to the direction of observation. It also follows that if the body is stretched, or compressed in the same proportion in both directions, *i.e.*, if $\frac{\Delta a}{a} = \frac{\Delta b}{b}$ no double refraction is to be observed.

Double Refraction in Gelatin

When gelatin is subjected to mechanical stresses it shows the same type of double refraction as ordinary glass, namely, that the direction of tension becomes the direction of minimum index of refraction, while the direction of compression is the direction of maximum index of refraction. The relation between elastic tension and double refraction in gelatin was studied in detail by Leick (11). He found

that the double refraction produced is proportional to the percentage elongation as well as to the concentration of the gelatin. He also determined the relation between mechanical stress and produced strain.

Leick's results are given in the following table:

Concentration C	Modulus of elasticity E	$\frac{E}{C^2}$	$\frac{E}{C}$
	Hard gelatin		
10.0	242	2.4	24.2
18.6	978	2.8	52.6
30.0	1545	1.7	51.0
32.0	2157	2.1	67.0
45.0	2944	1.5	65.0
	Soft gelatin		
12.7	323	2.0	25.5
17.5	620	2.0	35.5
19.0	578	1.6	30.5
20.8	619	1.4	29.8
28.3	1000	1.2	35.3
31.7	1189	1.2	37.8
45.6	2327	1.1	51.0

The first three columns were copied from Leick's paper, while the last column was added by the writer. The table shows that Young's modulus of elasticity of gelatin for stretching increases with the concentration, just as double refraction does. But while double refraction increases in direct proportion to the concentration of gelatin, the modulus of elasticity increases, according to Leick's opinion, as the square of concentration, as shown in the third column. But a close examination of the last column shows that with the exception of the cases of 10 per cent for one kind of gelatin and the 45.6 per cent for the other kind, the value of E/C is approximately constant for each kind of gelatin, just as in the case of double refraction. Hence, it appears from Leick's data that the double refraction produced in gelatin by mechanical stress is proportional both to the stress applied and to the dimensional change in the gelatin, *i.e.*, to the produced strain. Leick studied also the effect of addition of salts on the double refraction as

well as on the modulus of elasticity of gelatin during stretching. This will be discussed later.

Double Refraction and Swelling of Gelatin

An extensive study of the double refraction which appears in gelatin during swelling was made by Quincke (12). His results may be summarized as follows:

When a solution of gelatin is allowed to set and care is taken to prevent evaporation and drying the formed gel shows no trace of double refraction. If the gel is now placed in a solution in which it swells it becomes doubly refractive. The amount of double refraction is proportional to the *rate* of swelling and it disappears completely when the gel stops swelling. Gels in the form of cylinders or spheres on swelling show the same kind of double refraction as sections of a positive spherulite; but when allowed to shrink in alcohol or glycerin they show the double refraction of a negative spherulite. Gels in the form of prisms when allowed to swell behave with respect to double refraction as if the outside layers of the gel were under tension, while the middle of the block shows negative double refraction, as if it were compressed.

The double refraction in all swollen gels lasts only a short while, from several hours to a few days depending on the size and shape of the block, and then disappears gradually.

Quincke explains this gradual disappearance of double refraction in gels when swollen as a gradual equalization between the compressive stresses in the middle layers of the gel and the tensile stresses in the outer layers. He sees in this phenomenon a proof of his theory of "Shaumstruktur" of gels.

Attempts were made to explain the gradual disappearance of double refraction in a swollen gel by the "relaxation theory" of Maxwell (13), according to which the elastic stresses in a viscous body tend to disappear, so that if the body is left to itself it gradually loses any internal stress, and the pressures are finally distributed as in a fluid at rest. This theory may hold for very dilute gels, but does not apply to concentrated ones at a temperature below 15°C., as observed by Hatschek (14).

It will be shown later that the real reason why double refraction

gradually disappears in a swollen gel lies in the tendency of a gel to swell equally in its three dimensions, no matter what the original shape of the block was. Double refraction exists in a swelling gel as long as there is a difference in the amount of swelling in the three dimensions of a block of gel. This is especially true of a thin strip of gelatin. The rate of swelling is most rapid on the flat surface of the strip, and at first the percentage increase in the dimension perpendicular to this surface is much greater than in the other two directions. Hence when observation is made through the narrow edge of the strip very striking double refraction may be observed. But gradually the other dimensions of the strip begin to increase until the percentage change is the same in all of them. As a result of this there is a gradual disappearance of double refraction. If it were possible by any means to control the swelling so as to make the gel swell in one direction only and prevent the swelling from spreading in the direction of the other two dimensions of the gel, the double refraction should then persist as long as the gel is swollen. After various trials the writer found that this could be accomplished most conveniently by casting gelatin in rectangular glass frames or on glass slides. In this way the gelatin swells only in one direction. The double refraction which takes place in the gel persists indefinitely as long as the gel is swollen, and, as will be shown later, is proportional to the swelling.

Technique and Apparatus

All the measurements were done in a cold room kept at 6°C. ± 1°C. Electrolyte-free gelatin was prepared from Cooper's unbleached powdered gelatin by the modified (15) Loeb method. A 2 per cent solution in distilled water became milky white when allowed to stand in a cold room. The pH of the solution was 4.95 and this was taken as the isoelectric point of this stock of gelatin.

Various solutions were made up by dissolving definite weights of air dried gelatin in weighed amounts of water. Corrections were made for the moisture content of the gelatin which happened to be 15 per cent. The concentrations of the gelatin were often checked by drying weighed samples for 24 hours at 100° to 110°C. All weights of gelatin in this paper refer to gelatin dried in this way. The gels were prepared by heating the mixtures of gelatin and water to 50°C., were kept at this temperature until the gelatin grains were completely dissolved, and then put into forms and allowed to set for 20 to 24 hours in the cold room. Two forms of blocks of gelatin were employed in this work. Most of the experiments were done on gels cast in glass frames, but a number of experiments were done also on gels cast on glass slides.

Gels in Glass Frames.—The frames were made from narrow strips of glass about 3 mm. wide cut from microscopic slides 1 mm. thick. The strips were bent into rectangular frames 20 mm. × 10 mm. by means of the blast flame and sealed. Examination with the polarizing microscope showed strain in the frame only at the corners where the bending and sealing had taken place, while in the middle of the longer sides no trace of strain could be detected. The sharp edges of the frame were ground and the frames were marked and numbered. Two lines about 2 mm. apart were drawn with glass ink in the middle of both long sides of each frame. The inner width of the frames at the middle were measured to 0.01 mm. by means of a Zeiss measuring microscope CG. As the sides were seldom absolutely parallel, it was necessary to measure the width at the top and bottom of the frame and use the average of both measurements. The two widths never varied more than 0.2 mm.

The exact weight of the frames was also determined. The frames were placed on slides, filled with liquid gelatin and covered with thin slides. These were then put in rubber stoppered tubes containing some water so as to prevent any possible drying of the gelatin and allowed to remain in the cold room for 20 to 24 hours. The frames containing the gel were afterwards removed from the slides by means of a razor blade, cleaned of any adhering gelatin on the outside walls, weighed, and put into flasks containing various solutions. Examination of the gel in the frame at this stage showed very little if any double refraction. The gels in the frames were examined for double refraction at various lengths of time, usually after about 5, 10 and 20 days.[4] The beam of light from the polarizing Nicol was made to pass through the full width of the frame and the microscope was focussed on the space in the side of the frame between the two marked lines. Thus observations were always made through the same depth and the same location of the gel. The measurements of double refraction were made on the gels while immersed in the solutions in which they were allowed to swell.

At the same time the amount of swelling was determined by drying the frames carefully with filter paper and weighing.

Gels on Glass Slides.—Rectangular glass discs 25 mm. × 15 mm. were cut from microscopic slides, numbered and weighed. These were placed on regular slides supported by small strips of glass about 1 mm. thick, thus leaving an equidistant space of 1 mm. height between the small and larger slides. Liquid gelatin was then allowed to flow into the space between the slides by means of a pipette. The gelatin filled up the space readily, assisted by surface tension.

The slides were then put away in stoppered moist tubes to set for 20 to 24 hours. The gelatin was afterwards carefully removed from the larger slide by means of a razor blade, but allowed to adhere to the smaller slide. A rectangular steel die of 20 mm. × 10 mm. opening was pressed against the gelatin on the small slide

[4] It was found necessary to put a crystal of thymol in every solution in order to prevent mould growth on the gelatin.

thus enabling us to obtain a very uniform rectangular disc of gelatin of definite dimensions and parallel edges on the small slide. The exact length and width of the gel discs on the slides were measured to 0.01 mm. by means of the measuring microscope. It was found very convenient to direct a fine but strong horizontal beam of light, of the kind used in ultramicroscopy, on the gelatin during these measurements so as to produce a Tyndall cone in the gel. This facilitated enormously the focussing on the edges of the disc. The dimensions were determined both at the exposed surface of the gel disc as well as at the surface in contact with the glass slide. The gels were then weighed and put into the swelling medium as in the case of the gels in frames. The optical observations in this case were made through the middle of the long edge of the disc of gelatin.

The two kinds of blocks of gel when placed in water swell differently. The gels in the frames swell in one direction only, namely in thickness, so that the percentage change in total volume of the gel is the same as the percentage change in thickness. The gels which are cast on slides swell largely in thickness, but at the same time the other two dimensions change slightly too, the gel finally appearing as a thin frustum of a rectangular pyramid, with the smaller base adhering to the glass. In the case of the gels cast in the glass frames it is enough to weigh the gel from time to time, in order to be able to determine the changes in its linear dimensions; while in the case of the gels cast on glass slides it is necessary to measure actually the changes in the three dimensions of the gel. As it will be seen later the measured double refraction per unit change in linear dimension is identical in both forms of gels.

The apparatus for measuring double refraction consisted of a Leitz Babinet compensator fitted into an ordinary microscope which was provided with a polarizing Nicol in the substage, and with an analyzer over the eyepiece of the compensator. The microscope was held in horizontal position. A rectangular cell 3 cm. long, 2 cm. wide and 2 cm. high, made of plane optical non-strained glass was fastened by clips to the stage of the microscope and could be moved into various positions in the vertical plane by means of micrometer screws. The gels on the slides or in the frames were placed in the cell in a flat position and covered with the solution in which they had been allowed to swell. The cell was then moved on the stage into such a position that the microscope could be focussed on the middle point of the front edge of the gel. The beam of light was thus made to pass through the full width of the gelatin disc.

A Cooper-Hewitt mercury quartz lamp, the Lab-Arc, was used as a source of illumination. It was provided with two glass filters made by the Corning Glass

Works, a Didymium filter and a G 34-y filter, which allowed only the mercury 5461 Å.U. green light to pass through. This was checked by means of a spectroscope.

The Babinet compensator as arranged in this work showed a positive displacement of the zero line to the left for double refraction corresponding to stretching of gelatin in a vertical direction and a negative displacement to the right when gelatin was compressed in the same direction.

A phase difference of one wave length of 5.461×10^{-4} mm. corresponded to 9.35 divisions of the scale.

Hence if δ represents the reading of the Babinet compensator in division then the phase difference

$$\Delta p = \frac{\delta}{9.35}$$

Substituting in the formula $\Delta_p = \frac{s}{\lambda_0}(n_2 - n_1)$ (Equation 1) values for Δ_p and λ_0 we get

$$n_2 - n_1 = \frac{\delta}{9.35} \times \frac{5.46 \times 10^{-4}}{d} = 5.84 \times 10^{-3} \frac{\delta}{s}$$

where s is equal to the width of the gelatin disc in millimeters. The value of $n_2 - n_1$ is the double refraction produced by the algebraic sum of the elastic strains in the gel in directions lying in a plane perpendicular to the width, *i.e.*, by changes in the thickness and in the length of the gelatin disc. In the case of gels cast in frames the only possible change is in the thickness of the disc which is the same as the change in the total volume of the disc of gel.

Let W_0 be the weight in gm. of the gel when set
V_0 its volume when set
D_g the density of the gel
D_w the density of the water = 1
W_1 the changed weight of the gel after swelling

When the swelling takes place in a very dilute salt solution we have

$$\frac{\Delta V}{V_0} = \frac{(W_1 - W_0)/D_w}{W_0/D_g} = \frac{\Delta l}{l_0} \qquad \text{(Equation 2)}$$

where $\frac{\Delta V}{V_0}$ and $\frac{\Delta l}{l_0}$ are the fractional changes in volume and thickness.

When concentrated salt solutions are used in the outside solution the fractional change in the volume of the gel is

$$\frac{\Delta V}{V_0} = \frac{(W_1 - W_g)/D_s - (W_0 - W_g)/D_w}{W_0/D_g} \qquad \text{(Equation 3)}$$

where W_g = weight of dry gelatin in the gel and D_s = density of salt solution.

In deriving this formula it was assumed that the concentration of salt in grams per 100 gm. H_2O is the same inside of the gel as outside of it, which is true for high concentrations of salts as found by analysis (16).

The final concentration of gelatin in grams per cubic centimeters of H_2O or salt solution is

$$C = \frac{W_g}{(W_1 - W_g)/D_s} \qquad \text{(Equation 4)}$$

The double refraction per unit change in dimension and per unit concentration for gels cast in frames is

$$\alpha = \frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \qquad \text{(Equation 5)}$$

which may be termed the optical modulus of elasticity of a gel containing 1 gm. of dry gelatin per cubic centimeter of H_2O or salt solution.

When gelatin is cast on slides it swells not only in thickness but also in length, and the double refraction is a function of the value μ where

$$\mu = \frac{\Delta t}{t_0} - \frac{\Delta l}{l_0}$$

It was found impossible to measure accurately the change in thickness of the gels, but there was no difficulty in measuring the changes in length and width. The method employed to determine the change in thickness was to determine the fractional change in volume $\frac{\Delta V}{V_0}$ in

the same way as in the case of the gels in the frame. Exact determinations were also made of $\frac{\Delta l}{l_0}$ and $\frac{\Delta w}{w_0}$, *i.e.*, the fractional changes in length and width.

The value of $\frac{\Delta t}{t_0}$, the fractional change in thickness, was then calculated from the formula

$$\frac{\Delta t}{t}\left(1+\frac{\Delta l}{l}+\frac{\Delta w}{w}+\frac{\Delta l}{l}\times\frac{\Delta w}{w}\right)=\frac{\Delta V}{V}-\left(\frac{\Delta l}{l}+\frac{\Delta w}{w}+\frac{\Delta l}{l}\times\frac{\Delta w}{w}\right)$$

When $\frac{\Delta l}{l}$ and $\frac{\Delta w}{w}$ are very small fractions of a unit then the formula becomes

$$\frac{\Delta t}{t}=\frac{\Delta V}{V}-\left(\frac{\Delta l}{l}+\frac{\Delta w}{w}\right)$$

It has been found necessary for accuracy in most of the experiments here to employ the complete formula. The optical modulus of elasticity in the case of the gels on slides is then

$$\alpha=\frac{n_1-n_2}{\mu C} \qquad \text{(Equation 6)}$$

Density Measurements

The density of various concentrations of gels at 6°C. is given in the following table:

Concentration in gm. gelatin per 100 cc. H_2O.....	5	6	8	10	12	14	16	18	20
Density of gel after 24 hrs. setting.....	1.019	1.022	1.028	1.033	1.039	1.045	1.051	1.056	1.060

The density of the various gels was determined by weighing discs of gels first in air and then in kerosene in the cold room. The discs of about 0.5 gm. weight were suspended from the beam of a balance by means of a blackened, fine platinum wire.

The density of the various salt and acid solutions used for the swelling experiments was determined in the cold room by means of a Westphal balance.

Precision and Errors

The weight and hence the volume of the gels can be determined with great precision. But there is always a possible error due to the difficulty of removing the last trace of the outside liquid by means of filter paper. Checks have shown that the values for V_1 and V_0 are correct to about 0.5 per cent. The percentage error in the double refraction measurements depends on the magnitude of the reading and is about 0.5 per cent for a magnitude of $(n_2 - n_1)$ of 5×10^{-5} and 5 per cent for $(n_2 - n_1)$ equal to 1×10^{-5}.

The length and width measurements have a percentage error of not more than 0.5 per cent. The probable percentage error in the final calculated value of the optical modulus of elasticity depends on the probable errors in the above mentioned measurements.

From the relation (Equation 5)

$$\alpha = \frac{n_2 - n_1}{\frac{V_0}{V_0} C} = \frac{(n_2 - n_1) V_0}{(V_1 - V_0) C}$$

it follows that the probable percentage error in α equals

$$p_\alpha = \sqrt{p^2_{(n_2 - n_1)} + p^2 V_0 + p^2 C + p^2_{(V_1 - V_0)}}$$

where p equals the probable percentage error in the various factors designated by the subscripts.

The probable percentage error in C is of the same magnitude as in V_0 or V_1.

An expression for the relation between the probable percentage errors in V_1 and V_0 and in their difference, *i.e.*, in $(V_1 - V_0)$ can be found as follows:

The probable *actual* error E in $(V_1 - V_0)$ due to errors e_1 and e_0 in V_1 and V_0 is

$$E = \sqrt{e_1^2 + e_0^2}$$

But

$$E = \frac{(V_1 - V_0) P}{100}, \quad e_1 = \frac{V_1 p_1}{100}, \text{ and } \quad e_2 = \frac{V_0 p_0}{100}$$

where P, p_1 and p_0 are the probable *percentage* errors in $(V_1 - V_0)$, V_1 and V_0. Hence

$$(V_1 - V_0) P = \sqrt{V_1^2 p_1^2 + V_0^2 p_0^2}$$

Dividing through by $V_1 - V_0$ we get

$$P = \sqrt{\left(\frac{V_1}{V_1 - V_0}\right)^2 p_1^2 + \left(\frac{V_0}{V_1 - V_0}\right)^2 p_0^2}$$

This expression shows that the probable percentage error in $V_1 - V_0$ increases enormously as $V_1 - V_0$ approaches zero.

Thus for small magnitudes of swelling both the errors in the double refraction and in the value of $\frac{\Delta V}{V_0}$ contribute to a possible enormous error in the calculated optical modulus of elasticity.

Swelling of Thin Unsupported Discs of Gelatin

Thin rectangular discs of isoelectric gelatin were cast on glass slides and allowed to set in a cold room for 24 hours. The gels were afterward removed from the glass slides, weighed, measured, and put into 100 cc. $\frac{M}{1000}$ acetate buffer pH 4.95.

TABLE I

Rate of Change in the Three Dimensions of a Thin Rectangular Disc of Isoelectric Gelatin on Swelling in $\frac{M}{1000}$ *Acetate Buffer pH 4.95. Concentration: 20 Gm. of Gelatin in 100 Cc. Solution. Weight 0.311 Gm., Length 21.25, Width 9.45 Mm.*

Time in hrs.	1.25	2.25	3.75	6.0	24
Percentage increase in volume	10.30	16.00	20.10	23.40	27.50
" " " length	2.55	4.55	5.70	6.50	8.00
" " " width	1.90	5.45	5.60	6.40	7.95
" " " thickness (calculated)	5.70	5.30	7.50	9.00	9.20

Table I shows the rate of change in the various dimensions of the gelatin. The gel changed most rapidly in thickness, which in about 1 hour gained almost 60 per cent of its total increase while the other two dimensions gained in the same time only about 25 per cent of the final gain. But as the swelling progressed the rate of swelling in thickness diminished while the length and width continued to increase constantly until finally the percentage gain was the same in all three dimensions.

Table II shows the final percentage change in the three dimensions of unsupported discs of gels of various concentrations. In practically all cases there was no difference in the final percentage change of the various dimensions. The double refraction in these gels although very marked at the beginning of swelling disappeared entirely after

24 hours. It is obvious that the reason for its disappearance lies in the fact that the elastic strain became equal in all directions.

TABLE II

Swelling of Unsupported Thin Discs of Gels of Various Concentrations of Isoelectric Gelatin in $\frac{M}{1000}$ Acetate Buffer pH 4.95 at 6°C. Original Dimensions of Discs about 1.5 × 10 × 20 Mm.

Concentration in gm. gelatin per 100 gm. H_2O	5	6	8	10	12	14	16	18	20
After 3 days in buffer solution									
Percentage change in length	−3.9	−4.7	−2.4	0	+1.8	+3.0	+5.0	+5.6	+6.9
" " " width	−5.3	−5.3	−2.5	−.5	+1.4	+2.8	+5.2	+6.6	+7.6
" " " thickness	−8.8	−4.4	−2.8	−1.2	+.6	+4.2	+3.7	+6.0	+7.2

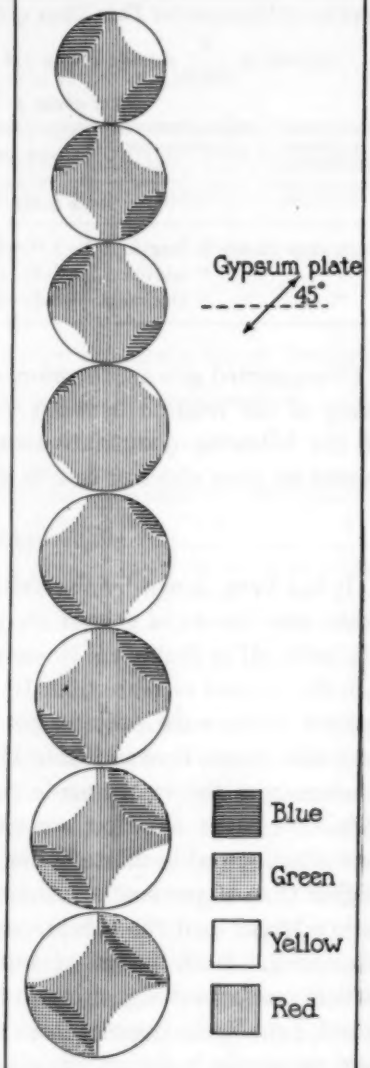
Unsupported gels are therefore entirely unsuitable for a quantitative study of the relation between double refraction and swelling; hence all the following quantitative experiments were made with gels supported on glass slides or cast in glass frames.

Effect of Concentration

It has been shown by the writer in his paper on syneresis (17) that when solid blocks of gelatin are placed in cold dilute buffer solution of the same pH as that of the isoelectric point of the gelatin, only those of a gelatin content of more than 10 per cent swell, while those of a lower gelatin content not only do not swell but actually lose water. This was also shown here in Table II. In working out the theory of this phenomenon the writer made the assumption that at setting all the gels no matter of what concentration are under no elastic stress, but when placed in dilute buffer solution the gels of a concentration of higher than 10 per cent are under tensile stresses due to swelling, while below 10 per cent the gels are under compressive stress resulting from shrinking. Both forms of internal stresses act against the forces which cause swelling on the one hand and shrinking on the other hand, namely the osmotic force due to the soluble ingredient of gelatin and the strain in the micells of the gelatin.

With the object in view of finding out whether double refraction

Concentration of gelatin in gm. per 100 gm. H_2O	Percentage increase in weight	Appearance of gels in polarizing microscope over gypsum plate R_1
5	-14.4	
6	-12.3	
8	-6.6	
10	-1.3	
12	+ 4.1	
14	+ 6.6	
18	+15.6	
20	+18.0	



TEXT–FIG. 1. Interference colors in cylinders of gels of various concentrations.

would show the difference in the form of stress in blocks of gelatin of various concentrations the following series of experiments were made.

1. Observation of Interference Colors in Cylinders of Various Gelatin Content.—Gelatin solutions of various concentrations were allowed to set for 24 hours in short glass tubes of about 8 mm. diameter, stoppered at both ends. The gels were removed next day by means of a cork borer and cut into short cylinders of about 10 mm. high and 5 mm. in diameter. These were weighed and put into 100 cc. $\frac{M}{1000}$ acetate buffer pH 4.95.

TABLE III

Swelling and Double Refraction of Blocks of Various Concentrations of Gelatin Cast in Frames. Outside Solution $\frac{M}{1000}$ *Acetate Buffer pH 4.95*

Gm. gelatin per 100 gm. H_2O	12	14	16	18	20
After 20 Days:					
$100\frac{\Delta V}{V_0}$	5.00	8.84	13.16	17.33	20.30
$100\,C$	11.40	12.75	13.90	15.00	16.15
$(n_2 - n_1) \times 10^3$	2.29	4.20	6.75	10.22	11.54
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	4.01	3.72	3.68	3.93	3.53

Text-fig. 1 shows schematically the colors in the gels after 36 hours, when observed through the top of the cylinder between crossed Nicols in combination with a gypsum test plate R_1. Ordinary light was used as a source of illumination.

The difference in the position of the blue and yellow colors in the gels of a higher or lower concentration than 10 per cent is very striking. In the 10 per cent gel there is very little blue or yellow color except red due to the gypsum plate.

2. The Quantitative Relation Between Double Refraction and Swelling or Shrinking in Various Concentrations of Gelatin.—Tables III and IV give the results of observations on gels cast on slides as well as on gels cast in frames. The frames are not suitable for gels which shrink when placed in water, since the gels become detached from the glass.

TABLE IV

Swelling and Double Refraction of Blocks of Various Concentrations of Gelatin Cast on Slides

V = volume, l = length, w = width, t = thickness

Gm. gelatin per 100 gm. H_2O	5	6	8	12	14	16	18
After 24 Days:							
$100\frac{\Delta V}{V_0}$	−21.3	−19.8	−11.0	+4.87	+10.05	+16.10	+22.3
$100\frac{\Delta l}{l_0}$	−2.70	−1.90	−1.04	+.24	+.64	+.85	+1.39
$100\frac{\Delta w}{w_0}$	−4.67	−5.10	−2.74	+.10	+1.27	+2.54	+2.96
$100\frac{\Delta t}{t_0}$	−13.75	−11.86	−6.90	+4.65	+8.55	+12.30	+17.15
$100\mu = 100\frac{\Delta t}{t_0} - \frac{\Delta l}{l_0}$	−11.05	−9.96	−5.86	4.41	+7.91	+11.45	+15.76
$100\,C$	6.40	7.54	9.00	11.40	12.53	13.57	14.40
$(n_3 - n_1) \times 10^3$	−2.54	−2.98	−2.35	+1.79	+4.03	+6.08	+9.00
$\frac{n_2 - n_1}{\mu C} \times 10^3$	3.59	3.94	4.45	3.56	4.06	3.92	3.96
Some of the gels were removed from the glass and left in the $\frac{M}{1000}$ acetate buffer for 8 days longer							
Final Measurements:							
$100\frac{\Delta V}{V_0}$				+5.1		+20.9	+27.2
$100\frac{\Delta l}{l_0}$				+1.00		+4.27	+5.36
$100\frac{\Delta w}{w_0}$				+1.05		+5.08	6.74
$100\frac{\Delta t}{t_0}$				+2.9		+10.3	+13.1
100μ				+1.9		+6.0	+7.7
$100\,C$				11.4		13.0	13.7
$(n_3 - n_1) \times 10^3$				+.92		+3.24	+4.09
$\frac{n_2 - n_1}{\mu C} \times 10^3$				4.25		4.15	3.88

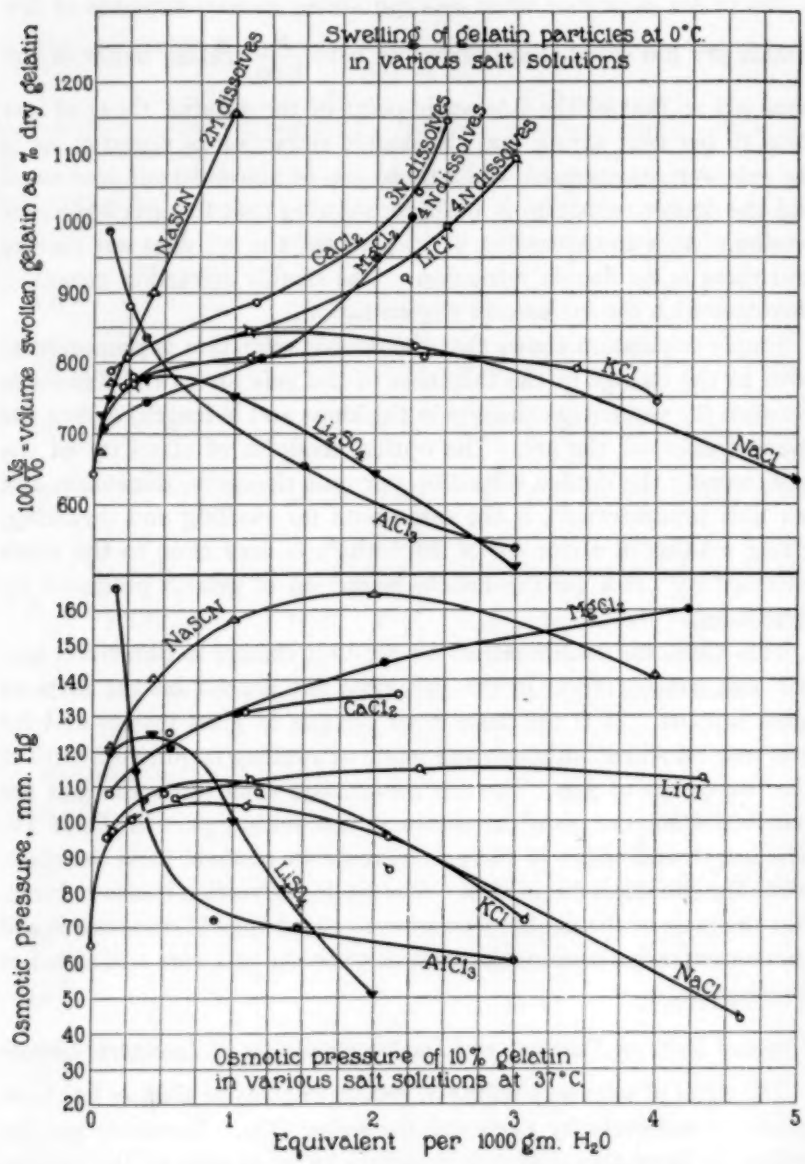
The tables show that when gels containing various amounts of dry gelatin per 100 cc. H_2O are placed in cold $\frac{M}{1000}$ acetate buffer of the same pH as that of the isoelectric point of the gelatin, those of less than 10 per cent shrink and the double refraction is negative, as if the gels were compressed, while those gels of above 10 per cent swell and the double refraction is positive, showing that the gels are under tension. At a concentration of 10 per cent the gel does not change and there is no double refraction. The double refraction generally increases with the increase in concentration.

Simple calculation shows that the double refraction is proportional both to the change in the thickness of the gels (or to the difference between the percentage changes in thickness and in length) and to the concentration of the gel. The optical modulus of elasticity of the gels, namely the double refraction per unit change in dimension and per unit concentration, is the same both for swelling and shrinking, giving a value of about 4.0×10^{-3} which is very close to the value obtained by Leick for the double refraction in gelatin produced by stretching.

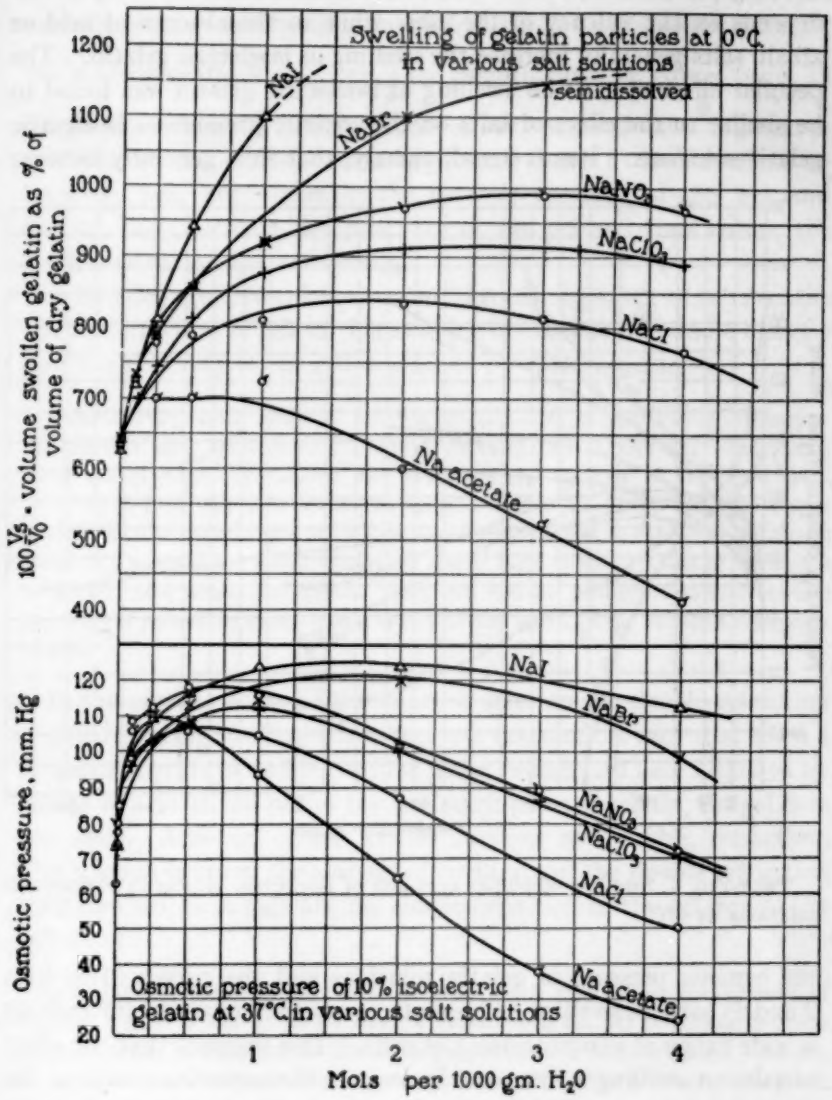
This value for double refraction per unit change in dimension and per unit concentration in the gels does not depend on the form of glass support. It is the same both for gels in glass frames and for gels cast on slides, although the mode of swelling is quite different in the two forms of gels. It does not change even when the gels are removed from the glass, as shown in the second part of Table IV. The length and width of the gels increase on removal from the glass, while the thickness diminishes. The double refraction is also reduced. But the ratio of the double refraction to the change in dimensions and the concentration remains the same as when the gels were still attached to the glass.

Effect of Salts on Swelling and Double Refraction of Isoelectric Gelatin

The effect of salts on swelling of gelatin in acids or alkalies has been studied extensively by Loeb and the writer (18). Northrop and the writer (6) have also studied in detail the effect of salts on the swelling of isoelectric gelatin in the absence of either acid or alkali. The effect of salts is quite different in the two cases. Salts always depress the

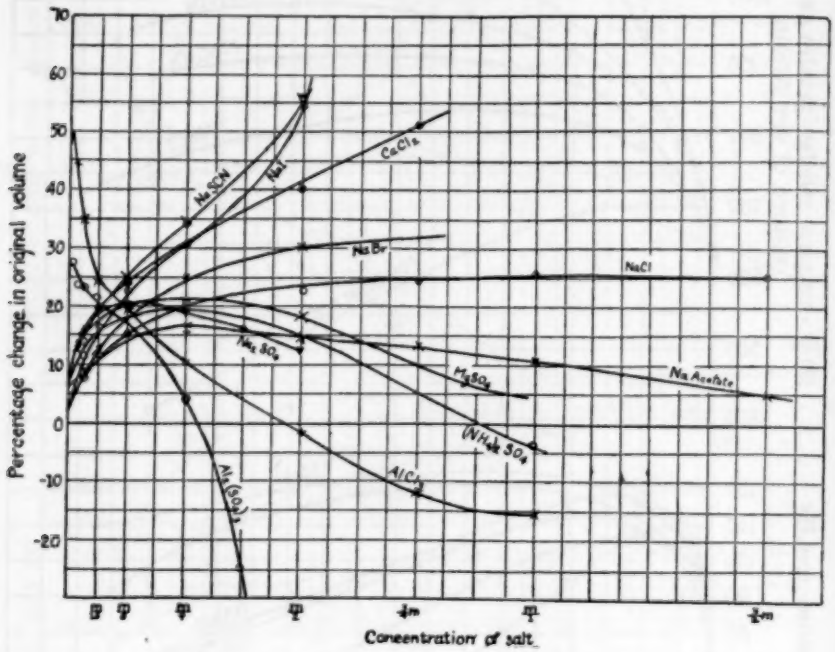


TEXT-FIG. 2. Comparison of osmotic pressure and swelling of gelatin in various salt solutions. (Reprinted from *J. Gen. Physiol.*, 1926, **8**, 322.)


Swelling of gelatin particles at 0°C.
in various salt solutions
semidissolved
NaI
NaBr
NaNO3
NaClO3
NaCl
Na acetate
100 Vs/Vo = volume swollen gelatin as % of volume of dry gelatin
Osmotic pressure, mm. Hg
Osmotic pressure of 10% isoelectric gelatin at 37°C. in various salt solutions
Mols per 1000 gm. H2O

TEXT-FIG. 3. Comparison of osmotic pressure and swelling of gelatin in various salt solutions. (Reprinted from *J. Gen. Physiol.*, 1926, **8**, 323.)

swelling produced by acid or alkali, and the amount of depression depends on the valency of the ions, while in the absence of acid or alkali, salts generally increase the swelling of isoelectric gelatin. The peculiar effect of salts on swelling of isoelectric gelatin was found to be similar to the effect of salts on the osmotic pressure of isoelectric gelatin solutions. It was found, namely, that salts generally increase



TEXT-FIG. 4. Swelling of 10 per cent gels of isoelectric gelatin in various salt solutions at 6°C.

the osmotic pressure of gelatin solutions and the curves (Text-figs. 2 and 3) both for swelling and osmotic pressure run parallel throughout a wide range of concentrations of salts. This suggests that the effect of salts on swelling is due to an increase in the osmotic pressure of the gel caused either by increase in the concentration of the osmotically active soluble component of the gel (2) or by an unequal distribution of ions through the formation of complex ions with the gelatin (16).

On the other hand, it was found that in the case of such salts as

NaSCN, NaI, NaBr, LiCl and $CaCl_2$ the parallelism between the osmotic pressure and swelling curves holds only for low concentrations, while at concentrations above $\frac{M}{2}$ the osmotic pressure curves begin to drop with increase in concentration of salt, while the swelling curves continue to rise. These salts apparently have a double effect on swelling, first, they increase the osmotic pressure of the gel, and second, they decrease the elasticity of the gel and thus reduce the resistance of the gel to swelling.

A detailed study was hence undertaken on the effect of salts on the double refraction in gels during swelling in order to determine directly how the elasticity of the gels is affected by various salts.

Some studies on the effect of salts on the elasticity of gels during mechanica deformation have been made by several investigators. Fraas (19) found that NaCl makes gelatin set slower and decreases the tenacity of the gel. Leick (11) found that chlorides lower the specific double refraction and the elastic modulus of stretching of gels while sodium sulfate produces no change in the elastic properties of gelatin. Leick employed rather high concentrations of salts, the minimum being about half molar. Sheppard and his collaborators (20) studied the effect of potassium alum as well as of acid and alkali on the modulus of rigidity of gels.

In the present study of the effect of salts on the double refraction of gelatin during swelling advantage was taken of the fact that when a 10 per cent gel is placed in distilled water, which had been adjusted by means of acid to the pH of the isoelectric point of gelatin, the gel does not swell. Addition of salt causes swelling and double refraction. The relation between the amount of swelling and the double refraction produced will thus indicate the elastic condition of the gel due to the salt alone.

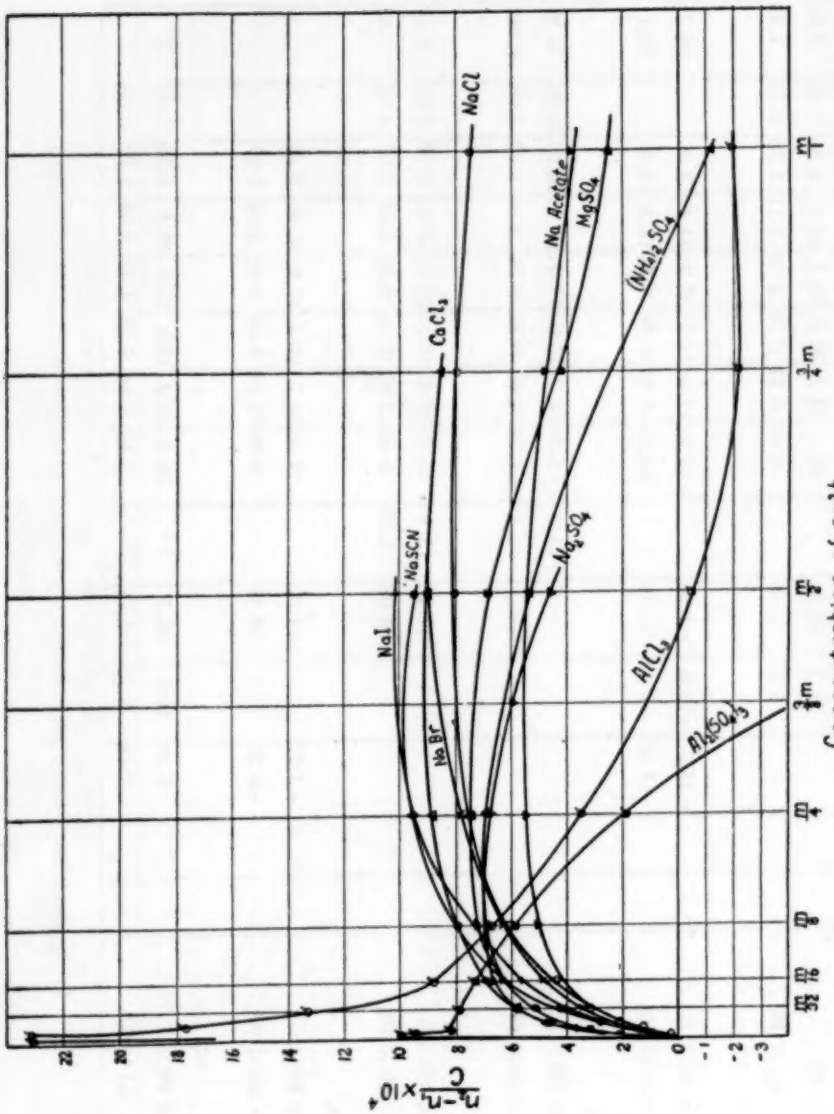
Salts generally change the pH of gelatin, which may affect the amount of swelling. But, as will be shown later, a variation in the pH of gelatin between 3.0 and 9.0 has no influence on the elastic properties of a 10 per cent gel. Hence, no extra precautions were taken to control the pH of the salt solutions used, except in the case of sodium acetate where acetic acid was added to bring the solutions to pH 4.95. In all other cases the weighed dry salt was dissolved in H_2O acidified to pH 4.95 with acid of the same anion as the salt, whenever possible, and the dilutions were made with this solution.

TABLE V

Swelling and Double Refraction of 10 Per Cent Gels in Various Salt Solutions at 6°C.
Gels Cast in Glass Frames. Weight of Gels about 0.8 Gm.
Measured after 19 to 24 Days

Concentration of salt solution.....	2 M	$\frac{3}{2}$ M	$\frac{M}{1}$	$\frac{3}{4}$ M	$\frac{M}{2}$	$\frac{3}{8}$ M	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$
NaCl:																
$\frac{\Delta V}{V_0} \times 10^3$.....	25.6	25.1	25.5	24.4	22.8		20.4	15.4	11.8	7.86	5.47	2.89	1.55	−.24		
$(n_2 - n_1) \times 10^5$.....	5.12	6.00	5.88	6.34	6.50		6.18	5.22	3.89	2.93	2.01	1.21	.29	−.36		
NaBr:																
$\frac{\Delta V}{V_0} \times 10^3$.....					30.3		25.0	18.0	13.0	8.83	5.03	2.20	.34	−1.0		
$(n_2 - n_1) \times 10^5$.....					6.80		6.16	5.28	4.27	3.13	1.92	.84	0	−.13		
NaI:																
$\frac{\Delta V}{V_0} \times 10^3$.....					54.2		30.9	22.7	16.3	11.7	6.34	3.72	2.22	.67		
$(n_2 - n_1) \times 10^5$.....					6.42		7.18	5.87	4.82	3.55	2.42	1.31	.40	.09		
NaSCN:																
$\frac{\Delta V}{V_0} \times 10^3$.....					55.8		34.3	24.8	17.9	11.2	6.30	2.50				
$(n_2 - n_1) \times 10^5$.....					5.96		6.86	6.20	5.37	3.74	2.63	1.02				
Na Acetate pH 4.95 (+ Acetic Acid):																
$\frac{\Delta V}{V_0} \times 10^3$.....		5.0	10.4	13.3	14.7	16.3	14.0	11.7	9.06	8.68	4.09	2.69	1.01			
$(n_2 - n_1) \times 10^5$.....		1.66	3.45	4.20	4.65	4.65	4.35	3.86	3.10	2.43	1.68	.82	.24			

$CaCl_2$:																
$\frac{\Delta V}{V_0} \times 10^2$				51.7	39.9		30.7	24.2	20.5	16.3	13.8	9.80	7.02	4.62	2.43	1.66
$(n_2 - n_1) \times 10^5$				5.53	6.38		6.61	6.31	5.70	4.89	4.17	3.33	2.56	1.89	.78	.48
$AlCl_3$:																
$\frac{\Delta V}{V_0} \times 10^2$			−15.3	−12.0	−1.54		+10.6	19.6	24.0	35.9	44.4	53.0		54.7	42.8	27.3
$(n_2 - n_1) \times 10^5$			−2.32	−2.43	−.46		+3.16	5.56	7.06	9.52	11.1	14.8		14.7	12.3	8.72
$Al_2(SO_4)_3$:																
$\frac{\Delta V}{V_0} \times 10^2$						−24.0	+3.80	17.3	22.0	23.0	23.4	26.8	26.0	26.9	26.3	22.6
$(n_2 - n_1) \times 10^5$						−8.3	+1.51	4.88	5.72	6.31		7.33	7.68	7.86	7.91	7.60
Na_2SO_4:																
$\frac{\Delta V}{V_0} \times 10^2$					12.1	15.9	18.3	20.4	17.7	16.1	12.6	8.70	5.56	4.29	.45	
$(n_2 - n_1) \times 10^5$					4.08	5.09	5.64	5.90	5.61	4.94	4.04	3.07	1.87	.85	.11	
$(NH_4)_2SO_4$:																
$\frac{\Delta V}{V_0} \times 10^2$			−3.72		+14.5		19.6	18.7	19.3	14.9	10.0	7.80				
$(n_2 - n_1) \times 10^5$			−1.22		+4.58		5.74	5.76	5.34	4.89	3.84	2.87				
$MgSO_4$:																
$\frac{\Delta V}{V_0} \times 10^2$			5.19	10.1	18.7		21.1	20.2	18.0	13.9	11.3	8.58	5.46			
$(n_2 - n_1) \times 10^5$			2.44	3.75	5.71		6.13	5.94	5.28	4.42	3.98	2.82	2.08			



TEXT-FIG. 5. Double refraction in 10 per cent gels of isoelectric gelatin produced by swelling in various salt solutions at 6°C. The observed double refraction was corrected for changes in the concentration of gelatin during swelling.

Table V gives a summary of the results obtained on the swelling and double refraction of 10 per cent isoelectric gels when put into various salt solutions. Experiments were performed both with gels in frames and gels on slides. The results are practically identical in both cases, except that for small values of swelling the results are subject to greater error in the case of gels on slides, where in addition to the weight the length and width of the gels also have to be measured, than in the gels in frames where only the weight of the gel is determined. The values for swelling and double refraction given in this table are those of gels cast in frames, and were taken after the gels had been allowed to remain in the salt solutions for about 20 days.

The plotted curves for swelling (Text-fig. 4) are practically identical with those observed by Northrop and the writer on the swelling of dry isoelectric gelatin granules, except for the curves for aluminum salts which show that in concentrations above $\frac{M}{4}$ both aluminum chloride and aluminum sulfate instead of causing swelling cause shrinking in 10 per cent gels at 6°C. This is also true in the case of $\frac{M}{1}$ ammonium sulfate.

The plotted curves (Text-fig. 5) for double refraction (corrected for the changed concentration[5] of the gelatin) run parallel to those of swelling for most of the salts. But in the case of salts which produce high swelling, namely, NaSCN, NaI and NaBr the double refraction curves do not rise with increase in concentration as fast as the swelling curves, which shows that in spite of the high swelling caused by these salts the internal stresses in these gels, as indicated by the double refraction observed, are not much different than in the gels kept in

[5] The final concentration of gelatin in the gels in gram per cubic centimeter of salt solution can be calculated from tabulated values of $\frac{\Delta V}{V_0}$ by means of the following equation:

$$C = \frac{1}{10.66\frac{\Delta V}{V_0} + 10.00}$$

which is easily derivable from Equations 3 and 4.

solutions at 6°C. The observed double refraction was corrected for changes in the concentration of gelatin during swelling.

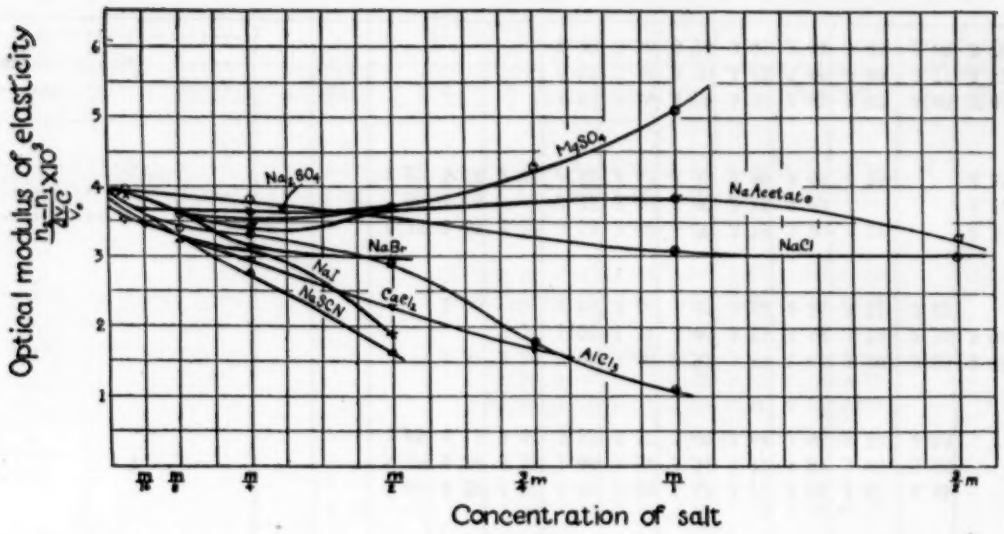
TABLE VI

The Optical Modulus of Elasticity of 10 Per Cent Gels in Various Salt Solutions at 6°C.

$$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^5$$

Concentration of salt solution	2 M	$\frac{3}{2}$ M	$\frac{M}{1}$	$\frac{3}{4}$ M	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$
NaCl:															
10 days	2.68	3.00	3.22		3.61	3.91	3.87	3.58	3.90	3.71	3.76				
24 "	2.54	3.03	2.95		3.53	3.68	3.95	3.69	3.99	3.88	(4.31)				
NaBr:															
14 days					2.92	3.09	3.70	3.87	3.83	3.98	4.09				
22 "					3.02	3.15	3.53	3.77	3.92	4.06	3.94				
NaI:															
13 days					1.96	3.04	3.42	3.46	3.55	4.16	3.73				
19 "					1.86	3.13	3.21	3.49	3.41	4.14	3.60				
Na Acetate:															
6 days		3.12	3.96		3.72	3.42	3.60	3.70	3.72	4.01	3.90				
16 "		3.49	3.67		3.69	3.36	3.56	3.70	3.75	3.89	4.29				
NaSCN:															
8 days					1.96	2.97	3.42	3.69	4.04		4.20				
16 "					1.83	2.82	3.30	3.63	3.85		4.20				
24 "					1.70	2.77	3.17	3.57	3.75		4.15				
Na_2SO_4:															
9 days					3.61	3.57	3.51	3.58	3.66		3.75				
22 "					3.79	3.68	3.52	3.76	3.60	3.63	3.86				

$(NH_4)_2SO_4$:															
7 days			3.64		3.65	3.72	3.87		3.91	4.28	4.11				
15 "			3.23		3.62	3.64	3.83		3.76	4.10	4.05				
22 "			3.17		3.64	3.55	3.70		3.77	4.24	4.00				
$MgSO_4$:															
7 days			5.04	4.26	3.87	3.61	3.68	3.63	3.77	3.66	3.87	4.18			
15 "			5.28	4.34	3.78	3.66	3.57	3.54	3.77	3.89	3.90	3.86			
22 "			4.95	4.13	3.67	3.55	3.57	3.49	3.64	3.96	3.59	4.03			
$CaCl_2$:															
5 days				1.57	2.36	2.99	3.40	3.52	3.59	3.62	3.89	3.65	4.35		
12 "					2.27	3.01	3.31	3.46	3.53	3.59	3.72	3.76	3.98		
21 "				1.66		2.86	3.28	3.39	3.53	3.46	3.75	3.92			
$AlCl_3$:															
5 days			1.35	1.65	2.20	3.55	3.42	3.90	3.75	3.70	4.25		4.35	3.98	4.06
13 "			.90	1.76		3.32	3.46	3.80	3.75	4.20			4.27	3.91	4.06
24 "			1.27	1.75	2.94	3.32	3.42	3.68	3.69	3.68	4.38		4.25	4.19	4.14
$Al_2(SO_4)_3$:															
8 days						4.58	3.19	3.29	3.36	3.41	3.62	3.68	3.82	3.64	4.03
21 "						4.15	3.35	3.20	3.42	3.46	3.46	3.77	3.77	3.85	4.16
30 "						4.38	3.36	3.26	3.41	3.44	3.43	3.69	3.78	3.76	4.08



TEXT-FIG. 6. The effect of salts on the elasticity of 10 per cent gels as calculated from double refraction measurements.

NaCl. These salts apparently lower the resistance of the gel to swelling by decreasing its elasticity.

Table VI and Text-fig. 6 give the values for the optical modulus of elasticity of the gels in the various salt solutions, namely, the value of double refraction per unit change in volume at unit concentration of gelatin, *i.e.*,

$$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C}$$

where C is the concentration of gelatin in gram per cubic centimeter of H_2O or salt solution at the time of measurement.

The curves show clearly how salts affect the elasticity of gels. In concentrations below $\frac{M}{8}$ most of the salts have practically no effect on the elasticity of gelatin, although they cause swelling. The optical modulus is the same here as the one obtained for swelling of various concentrations of gels in $\frac{M}{1000}$ acetate buffer pH 4.95. In concentrations higher than $\frac{M}{8}$ some salts change the elasticity of the gel, while others do not. Magnesium sulfate and also aluminum sulfate appear to increase the elasticity of the gels. Salts like NaSCN, NaI, $CaCl_2$ and NaBr which bring about rapid increase of swelling with increase in concentration exert a strong diminishing effect on the elasticity of the gels.

This difference in the effect of various salts on the optical modulus of elasticity of gels explains the discrepancy noted before between the osmotic pressure and the swelling curves for gelatin in the presence of salts, namely, that at low concentration the osmotic pressure curves for all salts rise with the concentration of salt, reach a maximum at about $\frac{M}{2}$, and then drop at higher concentrations of salt, while the swelling curves in the case of such salts as NaBr, NaI, NaSCN, $CaCl_2$ and others, continue rising with the increase in concentration. These salts lower considerably the elasticity and hence the resistance of the gels to swelling. The elasticity decreases rapidly with increase in

concentration of salt; hence the swelling continues increasing with the concentration until the gels become too soft to stand any swelling pressure and fall apart, or dissolve in very high concentrations of these salts.

Aluminum chloride in high concentrations produces a depression both in osmotic pressure and in swelling of gelatin, so that in solutions of above $\frac{M}{2}$ the gels shrink and lose in volume. The double refraction measurements show that the optical modulus of elasticity is decreased rapidly in concentrations above $\frac{M}{2}$. The resistance to shrinkage of the block is thus decreased with the result that the drop of the curve for swelling is more pronounced than the drop in the osmotic pressure curve.

The effect of salts on the elasticity of gelatin appears mostly in concentrations above $\frac{M}{8}$, while below that the elasticity remains constant. Loeb, in his studies on the effect of salt on swelling of gelatin in the presence of acid or alkali, limited himself mostly to low concentrations of salt, and thus by eliminating the elasticity variable was able to demonstrate clearly the valency rule in the action of salts on the swelling of gelatin caused by acid or alkali.

Swelling and Double Refraction of Gelatin in Acid and Alkali

The double refraction produced in 10 per cent gels when allowed to swell in various concentrations of HCl is shown in Table VII. The gels when placed in solutions of HCl in the concentration range of $\frac{M}{32}$ to $\frac{M}{2048}$ swell to such a high extent that they will not remain attached to glass when the gels are cast in frames or on slides. As seen from the table the optical modulus of elasticity of the gels immersed in the higher concentrations of HCl decreases rapidly both with the increase in concentration of acid used and with time. As a result of this the swelling of the gels in acid keeps on increasing with time and in high concentrations finally dissolves completely. The second rise in the

swelling curve of gelatin in acid above $\frac{M}{2}$ is due to the rapid drop in the elasticity of the gel.

TABLE VII

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of HCl at 6°C.

Concentration of HCl.....	$\frac{M}{1}$	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$		$\frac{M}{4096}$	$\frac{M}{8192}$
Final pH of outside solution...							3.45	3.95
After 9 Days:								
$\frac{\Delta V}{V_0} \times 10^2$.....	53.4	29.8	33.9	47.9	68.2	In $\frac{M}{32} - \frac{M}{2048}$	24.0	9.1
$C \times 10^2$.....	6.36	7.58	7.35	6.61	5.78	the gels swell	7.96	9.11
$(n_2 - n_1) \times 10^5$....	2.8	4.96	6.66	9.17	12.2	enormously	8.12	3.44
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$.....	.82	2.19	2.67	2.89	3.10	and fall out of the frames	4.24	4.19
After 17 Days:								
$\frac{\Delta V}{V_0} \times 10^2$.....	Enormous swelling, gels too soft to dry and weigh	44.2	43.3	55.6	77.8		28.1	9.90
$C \times 10^2$.....		6.80	6.86	6.28	5.45		7.69	9.00
$(n_2 - n_1) \times 10^5$....		3.99	6.60	9.25	12.40		8.84	3.83
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$.....		1.33	2.23	2.64	2.93		4.09	4.30
After 24 Days:								
$\frac{\Delta V}{V_0} \times 10^2$.....		53.7	51.5	62.4	85.4		30.2	11.1
$C \times 10^2$.....		6.36	6.46	6.00	5.22		7.56	8.95
$(n_2 - n_1) \times 10^5$....		3.56	6.60	9.30	12.45		9.13	4.05
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$.....		1.04	1.98	2.48	2.79		4.00	4.06

Table VIII shows the relation between swelling and the double refraction produced in gels placed in $\frac{M}{16}$ NaCl which contains various

TABLE VIII

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of HCl Made Up in $\frac{M}{16}$ NaCl

Concentration of HCl	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$	$\frac{M}{4096}$	$\frac{M}{8192}$	$\frac{M}{16384}$	0
Final pH of outside solution		1.3	1.6	1.9	2.7	2.9	3.3	4.0	4.4	4.8	5.0	5.2
After 6 Days:												
$\frac{\Delta V}{V_0} \times 10^2$	36.3	45.7	51.7	61.2	61.8	51.0	36.8	20.3	11.24	10.25	10.66	11.05
$C \times 10^2$	7.21	6.72	6.45	5.95	6.02	6.48	7.17	8.20	8.92	9.00	8.98	8.95
$(n_2 - n_1) \times 10^5$	8.41	10.10	11.26	13.15	13.00	12.20	9.64	6.72	3.97	3.40	3.75	3.70
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	3.21	3.29	3.37	3.61	3.50	3.70	3.65	4.05	3.96	3.68	3.92	3.74
After 14 Days:												
$\frac{\Delta V}{V_0} \times 10^2$	44.7	54.1	61.4	Excessive swelling and deformation of gel	73.3	60.4	44.5	24.4	13.60	11.75	12.10	12.70
$C \times 10^2$	6.78	6.34	6.05		5.60	6.08	6.77	7.93	8.72	8.97	8.86	8.80
$(n_2 - n_1) \times 10^5$	8.50	10.30	11.66		13.60	12.60	10.50	7.30	4.64	3.84	4.10	4.25
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	2.76	3.00	3.14		3.31	3.43	3.49	3.77	3.91	3.64	3.83	3.80
After 20 Days:												
$\frac{\Delta V}{V_0} \times 10^2$	46.6	55.7	63.5		75.2	62.0	45.9	25.1	13.8	11.9	11.9	12.70
$C \times 10^2$	6.68	6.24	5.96		5.54	5.96	6.70	7.88	8.71	8.88	8.88	8.80
$(n_2 - n_1) \times 10^5$	8.47	10.60	12.0		13.95	13.10	10.60	7.80	4.76	3.87	4.18	4.29
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	2.72	3.05	3.17		3.34	3.54	3.44	3.94	3.96	3.66	3.95	3.83

TABLE IX

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of H_2SO_4 at 6°C.

Concentration of H_2SO_4	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/512	M/1024	M/4096	M/8192	M/16384
Final pH of outside solution						1.5	1.7	2.0	2.8	3.0	3.6	3.8	4.0
After 6 Days:													
$\frac{\Delta V}{V_0} \times 10^3$	35.4	28.8	28.2	31.6	35.0	44.7	52.0	62.0	76.4	70.8	37.4	21.9	9.75
$C \times 10^3$	7.26	7.64	7.70	7.48	7.27	6.78	6.42	6.02	5.51	5.70	7.15	8.10	9.05
$(n_2 - n_1) \times 10^6$	3.86	5.70	6.30	7.41	8.58	9.46	11.60	13.40	14.06	14.35	10.06	7.15	3.62
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	1.50	2.59	2.90	3.13	3.37	3.12	3.48	3.59	3.34	3.55	3.77	4.03	4.10
After 16 Days:													
$\frac{\Delta V}{V_0} \times 10^3$	82.2	46.5	37.4	39.1	42.2	52.5	58.5	72.0	88.4	84.4	45.0	26.7	11.6
$C \times 10^3$	5.32	6.68	7.15	7.05	6.89	6.42	6.15	5.65	5.14	5.26	6.76	7.77	8.90
$(n_2 - n_1) \times 10^6$	1.08	4.93	6.36	7.60	8.64	9.60	11.70	13.8	14.50	14.55	11.45	8.36	4.02
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$	.25	1.59	2.38	2.78	2.97	2.86	3.25	3.40	3.14	3.28	3.78	4.01	3.89
After 22 Days:													
$\frac{\Delta V}{V_0} \times 10^3$	Gel fell apart	57.7	43.5	42.9	44.7	54.6	61.3	77.0	94.6	89.4	48.9	28.9	12.5
$C \times 10^3$		6.18	6.83	6.85	6.77	6.33	6.50	5.49	4.95	5.11	6.57	7.64	8.82
$(n_2 - n_1) \times 10^6$		4.49	6.54	7.85	8.85	9.82	11.85	14.25	14.70	14.25	11.80	8.85	4.27
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^3$		1.26	2.20	2.67	2.94	2.84	2.97	3.38	3.09	3.11	3.88	4.00	3.90

concentrations of HCl. The swelling produced by the acid is depressed considerably by the addition of NaCl and hence it is possible

TABLE X

Swelling and Double Refraction of 10 Per Cent Gels in Various Concentrations of NaOH Made Up in $\frac{M}{16}$ NaCl

Concentration of NaOH	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	$\frac{M}{2048}$
Final pH of outside solution	11.0	10.6	10.0	9.0	6.8
After 7 Days:					
$\frac{\Delta V}{V_0} \times 10^2$	53.9	48.4	43.2	35.3	22.7
$C \times 10^2$	6.34	6.60	6.83	7.26	8.04
$(n_2 - n_1) \times 10^5$	10.94	10.70	10.40	9.35	6.28
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^2$	3.21	3.35	3.52	3.65	3.44
After 15 Days:					
$\frac{\Delta V}{V_0} \times 10^2$	59.1	44.2	40.8	34.5	24.3
$C \times 10^2$	6.14	6.80	6.94	7.30	7.94
$(n_2 - n_1) \times 10^5$	11.10	9.78	9.40	8.92	7.16
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^2$	3.06	3.25	3.32	3.53	3.71
After 25 Days:					
$\frac{\Delta V}{V_0} \times 10^2$	61.9	42.4	38.5	33.7	24.3
$C \times 10^2$	6.02	6.88	7.06	7.35	7.94
$(n_2 - n_1) \times 10^5$	10.75	10.20	9.05	8.32	7.04
$\frac{n_2 - n_1}{\frac{\Delta V}{V_0} C} \times 10^2$	2.88	3.49	3.33	3.36	3.65

to make the observations over a wide range of pH. As in the case of pure acid the optical modulus of elasticity is reduced by the addition of acid but the effect of acid appears to become of significance only

at a pH of the acid below 2.0. At higher pH values the double refraction per unit increase in volume and per unit concentration is practically the same as in the absence of acid. The same is also shown in Table IX where are given data on the swelling and double refraction of 10 per cent gels in various concentrations of H_2SO_4. This acid also lowers the elasticity of gelatin, though it is not as effective as HCl. The effect of the acid on the elasticity of the gels practically disappears at a pH above 2.0.

Alkali affects the elasticity of gelatin in the same way as acids do, but the effect does not appear until the pH of the outside medium is not less than 10.0. This is shown in Table X. The slight drop with time in the swelling of the gels in $\frac{M}{256}$ NaOH and lower is due to the gradual lowering in the pH of the solution which is unavoidable in this range of pH in non-buffered solutions.

The results here on the effect of pH on the elasticity of the gel agree with those found by Sheppard and his coworkers and also by Scarth.[1]

SUMMARY AND CONCLUSIONS

Quincke's researches (1904) have demonstrated that when a 20 per cent gelatin gel is allowed to swell in water it gives rise to positive double refraction, as if the gel were under tensile stresses. If, on the other hand, the gel shrinks on being placed in alcohol it becomes negatively double refractive, as if it were compressed. But the double refraction as found by Quincke lasts only during the process of swelling or shrinking, and disappears as soon as the gel reaches a state of equilibrium.

This phenomenon was investigated here and it was found that the reason for the disappearance of the double refraction is due to the fact that at equilibrium the percentage change in the size of a gel is equal in all three dimensions and the strain is therefore uniform. Double refraction persists as long as there is a difference in the elastic strain in the three dimensions of the strained material.

It was found that when gels are cast on glass slides or in glass frames, so as to prevent swelling in certain directions, the double refraction produced by swelling at 6°C. persists permanently in the gel as long as it is swollen, and is proportional to the percentage change in the linear dimensions of the gel.

Gels made up of various concentrations of isoelectric gelatin of less than 10 per cent when placed in dilute buffer of the same pH as that of the isoelectric point of the gelatin shrink and give rise to negative double refraction, while gels of concentrations of more than 10 per cent swell and give rise to positive double refraction. The double refraction produced in either case when divided by the percentage change in the dimensions of the gel and by its changed concentration gives a constant value both for swelling and shrinking. This constant which stands for the double refraction produced in a gel of unit concentration per unit strain is termed here the optical modulus of elasticity since it is proportional to the internal elastic stress in the swollen gelatin.

It was found that the optical modulus of elasticity is the same both for gels cast on slides and in frames, although the mode of swelling is different in the two forms of gels.

Gels removed from their glass supports after apparent swelling equilibrium, when placed in dilute buffer, begin to swell gradually in all three dimensions and the double refraction decreases slowly, though it persists for a long time. But the double refraction per unit change in dimension and per unit concentration still remains the same as before, thus proving that the internal elastic stress as indicated by the double refraction is brought about by the resistance of the gel itself to deformation.

A study was also made on the effect of salts, acid and base on the double refraction of a 10 per cent gel during swelling. The experiments show that below $\frac{M}{8}$ salts affect very slightly the optical modulus of elasticity of the gel. At higher concentrations of salts the elasticity of the gel is reduced by some salts and increased by others, while such salts as sodium acetate and sodium and ammonium sulfates do not change the elasticity of the gels at all during swelling. The investigated salts may thus be arranged in this respect in the following approximate series: $CaCl_2$, NaI, NaSCN, NaBr, $AlCl_3$, NaCl, Na acetate, Na_2SO_4, $(NH_4)_2SO_4$, Al_2SO_4 and $MgSO_4$. The first five in the series decrease the elasticity while the last two in the series increase the elasticity of the gels during swelling. Acids and bases in higher concentrations exert a powerful influence on the reduction of the elasticity of the gel but in the range of pH between 2.0 and 10.0 the elasticity remains unaffected.

The general conclusions to be drawn from these studies are as follows:

1. Swelling or shrinking produces elastic stresses in gels of gelatin, tensile in the first case and compressive in the second case, both being proportional to the percentage change in the dimensions of the gel.

2. Unsupported gels when immersed in aqueous solutions swell or shrink in such a manner that at equilibrium the percentage change in size is equal in all three dimensions, and the stresses become equalized throughout the gel.

3. Gels cast on glass slides or in frames when immersed in aqueous solutions swell or shrink mostly in one direction, and give rise to unidirectional stresses that can be determined accurately by measuring the double refraction produced.

4. The modulus of elasticity of swelling gelatin gels, as calculated from the double refraction measurements, is the same both for compression and for tension and is proportional to the concentration of gelatin in the gel.

5. The modulus of elasticity of gels during swelling is affected only slightly or not at all by salts at concentrations of less than $\frac{M}{8}$ and is independent of the pH in the range approximately between 2.0 and 10.0.

6. Higher concentrations of salts affect the modulus of elasticity of gelatin gels and the salts in their effectiveness may be arranged in a series similar to the known Hoffmeister series.

7. Acid and alkali have a strong reducing influence on the elastic modulus of swelling gels.

8. The swelling produced in isoelectric gelatin by salts is due primarily to a change brought about by the salts in the osmotic forces in the gel, but in high concentrations of some salts the swelling is increased by the influence of the salt on the elasticity of the gel. This agrees completely with the theory of swelling of isoelectric gelatin as developed by Northrop and the writer in former publications.

9. The studies of Loeb and the writer on the effect of salts on swelling of gelatin in acid and alkali have been in the range of concentrations of salts where the modulus of elasticity of the gelatin is practically constant, and the specific effect of the various salts has been negligible as compared with the effect of the valency of the ions.

In concentrations of salts below $\frac{M}{4}$ or $\frac{M}{8}$ the Hoffmeister series plays no rôle.

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VARIATION OF BASAL METABOLIC RATE PER UNIT SURFACE AREA WITH AGE

By CURTIS BRUEN

(*New York, N. Y.*)

(Accepted for publication, April 8, 1930)

Basal metabolism empirically referred to surface area is theoretically related indirectly to its hypothetical determining factor. Definition of the basal metabolic rate per unit surface area as a function of age then constitutes proximate definition of the basal metabolic gradient.

The age gradient of calories per square meter per hour outlined by Du Bois is the foundation of the Du Bois standards. Base lines for males in the prime adult years for surface area computed from the Meeh formula (1) were located by averaging selected series of reported normals with normal controls (2, 3). A scatter diagram for males and females to the age of 24 years was plotted for similar series and smoothed curves drawn. A second scatter diagram for a series revised for surface area computed from the Lissauer formula (4) for infants and from the linear (5) or height-weight formula (6) for children and adults was plotted for the life span of males (7). New base lines were located for boys in later childhood, and for several age groups of men and women, and an average percentage decrement for females calculated (8). Readings taken from a gradient similar to that plotted for males and diminished a uniform percentage for females were tabulated as provisional age group standards (9). In the section of the gradient corresponding to the standards (Chart 1) a hyperbolic curve runs into a horizontal line for 20 to 30 years which runs into a sloped line to the upper age limit. Readings from a gradient in which the plateau of constant rate reaches to 35 years are assigned to age groups extending symmetrically to either side of them. The standards for females are in general calculated approximately 7 per cent below those for males of the same age groups (Table I).

The gradient and the standards are not consistent. The graph of

the gradient is in succession a hyperbolic curve, a horizontal line, and a sloped line. The graphs of the standards are series of horizontal lines (Chart 1). Within its range the gradient has two discontinuities,

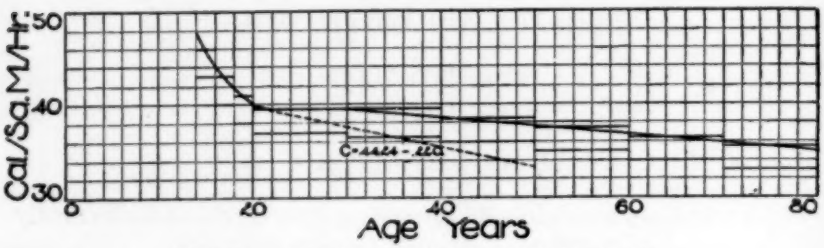

CHART 1. Basal metabolic rate (Aub-Du Bois).

TABLE I

Calories per Square Meter of Body Surface per Hour (Aub-Du Bois)

Sex	Age	Age group	Rate	Linear equation*	Exponential equation*
	yrs.	*yrs.*	*cal.*	$y = A + Bx$	$y = Ae^{Bx}$
Male	15	14–16	46.0	$\log C = 1.8488 - 0.01247\ a$	$C = 70.60\ e^{-0.0285\ a}$
	17	16–18	43.0		
	19	18–20	41.0		
	30	20–40	39.5	$\log C = 1.6301 - 0.00104\ a$	$C = 42.67\ e^{-0.0024\ a}$
	45	40–50	38.5		
	55	50–60	37.5		
	65	60–70	36.5		
	75	70–80	35.5		
Female	15	14–16	43.0	$\log C = 1.8329 - 0.01342\ a$	$C = 68.06\ e^{-0.0309\ a}$
	17	16–18	40.0		
	19	18–20	38.0		
	25	20–30	37.0	$\log C = 1.5972 - 0.00101\ a$	$C = 39.55\ e^{-0.0023\ a}$
	35	30–40	36.5		
	45	40–50	36.0		
	55	50–60	35.0		
	65	60–70	34.0		
	75	70–80	33.0		

* C = calories per square meter per hour, a = age in years, e = natural logarithmic base.

while the standards have seven and eight discontinuities for males and females respectively. While the successive segments of the gradient are expressible as a hyperbolic equation, a Fourier series, and a linear

equation, the partial intervals of the standards are expressible as Fourier series.

Linear regression equations calculated by Harris and Benedict (10) represent the adult gradient as

$$\text{For men} \ldots\ldots\ldots\ldots h_D = 1022.17 - 3.60\,a$$
$$\text{For women} \ldots\ldots\ldots\ldots h_D = 924.25 - 2.96\,a$$

in which h_D = heat production in calories per square meter per day, and a = age in years. The equation for the major part of the data (3) on which the middle stretch of the Du Bois gradient rests

$$h_D = 1061.81 - 5.25\,a$$

is drawn on a comparable hourly scale in Chart 1. Such equations do not necessarily define the variation of the basal metabolic rate with age. A linear regression equation represents the most probable value of the dependent variable only on the assumption and providing that it be a linear function of the independent variable. If this relationship is not functional but only empirical these equations do not define metabolic gradients but substitutive straight lines.

The basal metabolic rate per unit surface area may be treated as an exponential function of age. The logarithm of the rate is then a linear function of age. Thus the midpoints of the Du Bois standards for each sex plotted against age on an arithlog grid (Chart 2) lie approximately in two straight lines. The most probable constants for the first degree polynomial equations of their logarithms are calculated by the method of least squares. The linear equations of the logarithms are transformed into exponential equations of the rates (Table I). The fitted straight lines are plotted (Chart 2).

In a definitive revision of the Du Bois standards Boothby and Sandiford (11) give for each sex self-consistent series of means for halved age groups (Table II). Between the ages of 5 and 19, and 22 and 77, for males, and 5 and 17, and 18 and 77, for females, the yearly first order numerical differences as well as the first order logarithmic differences are approximately equal. But in order to obtain equations congruent with a rational mechanism of variation the logarithm of the basal metabolic rate per unit surface area is expressed as a linear function of age and the basal metabolic rate per unit surface

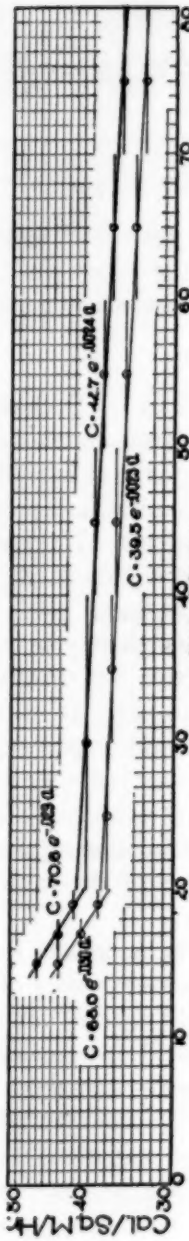

CHART 2. Basal metabolic rate (Aub-Du Bois).

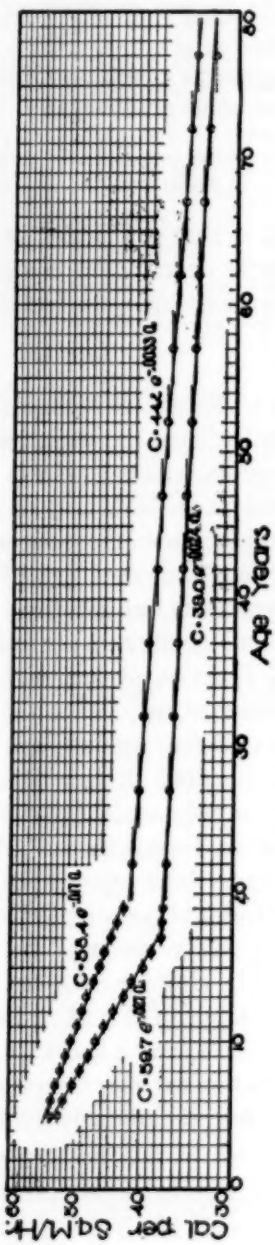

CHART 3. Basal metabolic rate (Boothby and Sandiford).

TABLE II

Calories per Square Meter of Body Surface per Hour (Boothby and Sandiford)

Sex	Age group*	Mid-value	Rate	Linear equation	Exponential equation
	yrs.	*yrs.*	*cal.*	$y = A + Bx$	$y = Ae^{Bx}$
Male	5	5	(53.0)	$\log C = 1.7667 - 0.00736\,a$	$C = 58.44\,e^{-0.0170\,a}$
	6	6	52.7		
	7	7	52.0		
	8	8	51.2		
	9	9	50.4		
	10	10	49.5		
	11	11	48.6		
	12	12	47.8		
	13	13	47.1		
	14	14	46.2		
	15	15	45.3		
	16	16	44.7		
	17	17	43.7		
	18	18	42.9		
	19	19	42.1		
	20–24	22	41.0	$\log C = 1.6450 - 0.00144\,a$	$C = 44.16\,e^{-0.0033\,a}$
	25–29	27	40.3		
	30–34	32	39.8		
	35–39	37	39.2		
	40–44	42	38.3		
	45–49	47	37.8		
	50–54	52	37.2		
	55–59	57	36.6		
	60–64	62	36.0		
	65–69	67	35.3		
	70–74	72	(34.8)		
	75–79	77	(34.2)		
Female	5	5	(51.6)	$\log C = 1.7759 - 0.01178\,a$	$C = 59.68\,e^{-0.0271\,a}$
	6	6	50.7		
	7	7	49.3		
	8	8	48.1		
	9	9	46.9		
	10	10	45.8		
	11	11	44.6		
	12	12	43.4		
	13	13	42.0		
	14	14	41.0		
	15	15	39.6		
	16	16	38.5		
	17	17	37.4		

TABLE II—*Concluded*

Sex	Age group*	Mid-value	Rate	Linear equation	Exponential equation
	yrs.	*yrs.*	*cal.*	$y = A + Bx$	$y = Ae^{Bx}$
Female—*Continued*	18	18	37.3	$\log C = 1.5909 - 0.00103\ a$	$C = 38.98\ e^{-0.0024\ a}$
	19	19	37.2		
	20–24	22	36.9		
	25–29	27	36.6		
	30–34	32	36.2		
	35–39	37	35.8		
	40–44	42	35.3		
	45–49	47	35.0		
	50–54	52	34.5		
	55–59	57	34.1		
	60–64	62	33.8		
	65–69	67	33.4		
	70–74	72	(32.8)		
	75–79	77	(32.3)		

* Class limits 7th month of preceding and 6th month of concluding year inclusive.

area as an exponential function of age. The formulas are calculated (Table II) and their graphs plotted (Chart 3). For children of each sex the residuals sensibly vary with age as though a subordinate factor which arose and subsided during this period were neglected. The residuals for adults are negligible.

The fitted straight lines for the provisional Du Bois standards may be compared with those for the Boothby and Sandiford revision. The discontinuities for males and females respectively occur at 19.1 and 19.0 years in the Du Bois standards and at 20.6 and 17.2 years in the Boothby and Sandiford revision. The fitted straight line for the Du Bois standards for younger males intersects that for the Boothby and Sandiford revision at 16.1 years and diverges from it from 2.5 per cent to −3.4 per cent between their common lower and upper extremes. The line for the Du Bois standards for adult males intersects that for the Boothby and Sandiford revision at 37.3 years and diverges −1.6 per cent to 4.5 per cent. The line for the Du Bois standards is for younger females from 8.2 per cent to 6.6 per cent, and for adult females 1.5 per cent to 1.8 per cent above that for the Boothby and Sandiford revision. The differences are varied and considerable.

The exponential formulas (Table II) define the gradients plotted by the Boothby and Sandiford standards. The rates graduated for continuous variation may be read off their graphs (Chart 4). Four-place logarithms for them may be computed from the linear formulas (Table II). Regular computations involving them can be made on alignments charts (12) or four variable straight line diagrams with logarithmic rectangular coordinates (13) so modified as to convert the serial variable into a continuous variable (14).

The not comparable, but self-consistent, determinations of minimum heat production per square meter of body surface per 24 hours for the basal conditions of infancy and childhood, of Benedict and Talbot (15), plot an age gradient for each sex from the end of the first week to

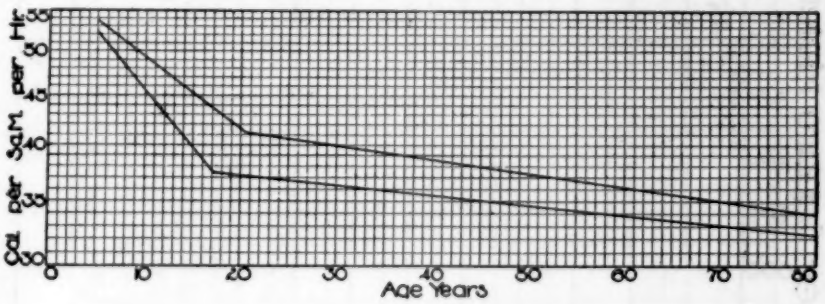

CHART 4. Basal metabolic rate.

the onset of the Boothby and Sandiford standards. The function represented by the smoothed curves of the medians (Charts 5, 6) is expressible as the difference of two negative exponential terms. The rising and falling age gradient of a physiological activity is the resultant of decrease and diminishing increase simultaneously proceeding exponentially (16, 17). On this ground Brody (17) deduced the equation

$$y = 56.7\, e^{-0.094t} - 32.0\, e^{-1.224\, t}$$

in which y = calories per square meter per hour, e = the natural logarithmic base, and t = years birth age, for the tentative Meeh formula gradient for males (7). Exponential equations for the decrease of the basal metabolic rate with age are derived for the parts of the curves where increase has become negligible and their

graphs projected to their origins. The residuals for decrease counteracted by increase are plotted with their signs changed and exponential equations for the negative of the increase of the basal metabolic rate

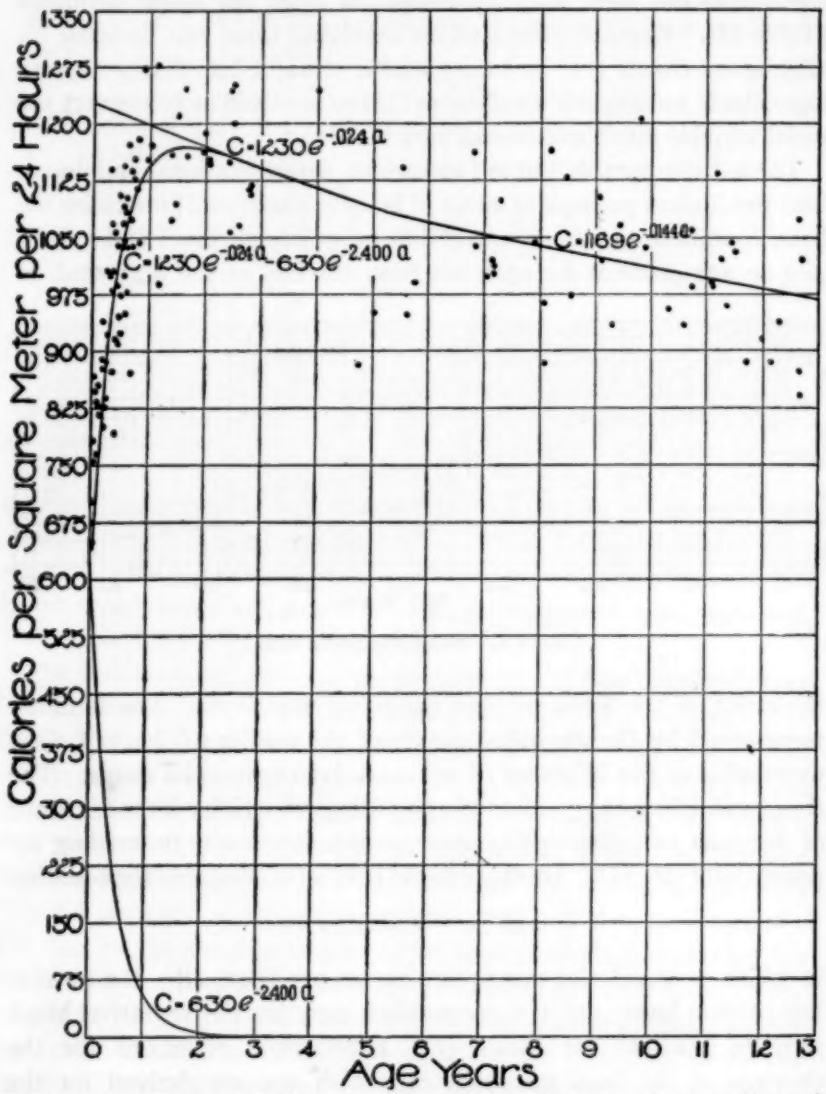

CHART 5. Basal metabolic rate of boys (scatter diagram after Benedict and Talbot).

with age derived for them. The gradient is defined by the term for decrease minus the term for negative increase (Charts 5, 6).

Thus the basal metabolic gradient is proximately defined throughout

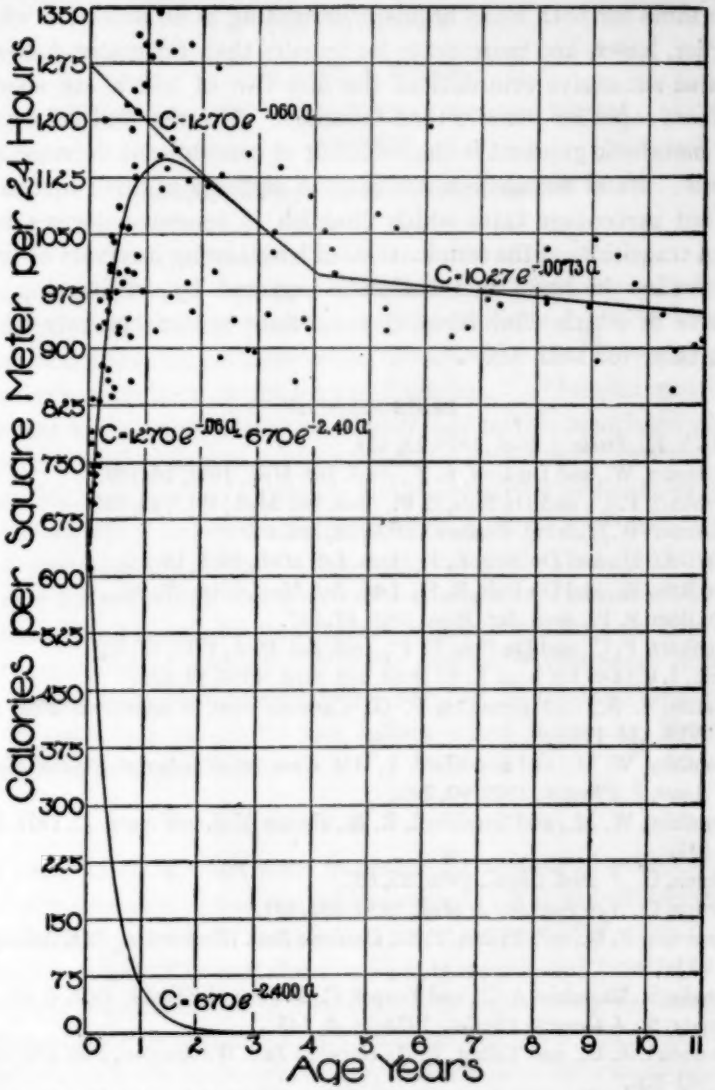

CHART 6. Basal metabolic rate of girls (scatter diagram after Benedict and Talbot).

post-natal life. The first day of extra-uterine readjustment and stabilization establishes basal metabolism at the level it maintains to the end of the first week (18). From this point an acceleration which is the same for both sexes begins, culminating in an inflection which is earlier, lower, and more acute for females than for males, followed by three successive retardations the first two of which are shorter and more rapid for females than for males. The rise and fall in the basal metabolic gradient is the resultant of concomitant decrease and increase. Basal metabolism decreases in each sex at three successive constant percentage rates which diminish in lessening degrees with abrupt transitions at the terminations of lengthening intervals of time. This decline in basal metabolism is opposed by an increase the negative of which diminishes at a constant percentage rate which is the same for both sexes.

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THE VALIDITY OF MINIMAL PRINCIPLES IN PHYSIOLOGY

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(Accepted for publication, February 7, 1930)

I

As a mechanism to explain the adaptation of physiological systems to the physical and chemical constraints placed upon them, certain theoretical physiologists have made the assertion that "physiology is essentially a problem in maxima and minima." This statement implies that a physiological process is such that certain associated physical or chemical processes are minimized or maximized. Murray has taken this statement as a premise and postulated a principle of minimum work which he applies to the problem of blood flow[1] and the angle of branching of arteries.[2] This work, in addition to being formally in error by neglecting the gravitational effect on Poiseuille's law of capillary flow,[3] has an inherent fallacy which arises from an improper use of reasoning by analogy. It is the purpose of this paper to point out certain features concerning the validity of minimal principles, and in particular to exhibit the fallacy which occurs in Murray's principle of minimum work.

II

In order to clarify just what is meant by a minimal principle, the necessary consequences of one will be stated. If it is asserted that a physiological process, Y, is such that a certain part of Y, say X, is a minimum, where X is contained in Y, it is meant that by simply imposing the requirement on X that it shall be a minimum, the complete configuration Y is deduced as a logical consequence. A concrete illustration is: If the hydrodynamical process of blood flow is considered, and if the assertion is made that the flow is such that the energy lost

in heat is a minimum, then by this restriction it must follow that the differential equations of flow are given as a logical consequence of minimizing the energy lost in heat, and that the flow is uniquely determined.

III

Formal Aspects of Minimal Principles

The problem of physiology or of any natural science which may be described to a high degree of approximation by assigning numbers to the independent variables over which it is assumed we have control, and observing and thus determining the numbers which are assigned to the dependent variables by the process in hand, is expressible analytically as follows:

A system of differential equations having all the variables and their derivatives present exists which completely describes the state of the process under consideration for any values of the independent variables. This is:

$$Y = 0 \tag{1}$$

If we exclude processes which go on at a constant rate, a sub-process X contained in Y which gives (1) as a consequence when minimized will be of the form:

$$X = \int_{t_1}^{t_2} f dt \tag{2}$$

where f is, say, a function of the energies of the system and t is the time. For example: Equation (2) might be the energy lost in heat in the interval $t_1 \leq t \leq t_2$ in the flow of blood. If Murray's assertion is true that the blood flow is such that the energy lost in heat is a minimum, then by minimizing (2) the equations of flow (1) must be a necessary consequence. This is what is known as the ordinary problem in the Calculus of Variations.

IV

The only justification for the postulation of the principle of minimum work is the reasoning by analogy with certain ideal physical systems. In order to show the actual steps of this method of reason-

ing it is perhaps well to state explicitly its import. If A has a set of properties, m, and X has a set, n, of these m properties, then the probability that X is A is the greater the nearer n and m approach equality.[4] If a single property of X and A are in contradiction, then the analogy is destroyed. It is well known that the equations of state of conservative dynamical systems are given by a minimal principle, the principle of Least Action.[5] The flow of blood, however, is dissipative, not conservative.* Hence the analogy is destroyed. Further than this, the author has shown in a paper soon to be published that, in general, the equations of state of a physical system are given by a variational principle (imposing a minimal or maximal requirement on some integral of a function of the energies of the system) if, and only if, the system is conservative.[6] The fallacy of the principle of minimum work as applied to the problem of blood flow is now apparent. Other applications of minimal principles to physiological systems should be made only after the most careful examination of the properties of the system under consideration and should not depend for their validity upon the improper use of the method of reasoning by analogy, and thus arrive at a theory which is incompatible with physical laws.

The author wishes to acknowledge his indebtedness for many suggestions to Professors W. J. Crozier and L. J. Henderson and to his colleagues in the Fatigue Laboratory.

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* A conservative system may be said roughly to be one in which the energy of the system is of one form: Mechanical, thermal, or electrical. A dissipative system has the property that some of the energy leaves the system in another form. For example, a mechanical system with friction is dissipative, since mechanical energy is changed to heat energy, which leaves the system in that form.

VARIATIONS IN THE POLARIZATION CAPACITY AND RESISTANCE OF THE SKIN*

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(Accepted for publication, March 27, 1930)

The name "psychogalvanic reflex" was given by Veraguth (1907) to certain electrical changes in the skin, although it had been noticed previously and had been studied by a number of investigators. Tarchanoff (1890) measured the bioelectric current of the skin but Fere (1888) and most workers have measured the electric resistance. In the opinion of the authors these two phenomena depend upon the same variables in the cells. Bio-electric currents and electric conductivity of cells in general both depend on certain variables in the cell surface and, although the chemistry of the process is not known, interesting electrical measurements on conductivity have been made by Fricke, Cole and others. It has been shown by experiments of Höber, Philippson, Fricke, McClendon, and others, that ions are free to move in the interior of the cell. With all possible assumptions of dielectric constant, it has been shown by Fricke and others that the impermeable layer is very thin on the surface of some resting cells; in fact, it is not greater in thickness than the length of a fatty-acid molecule.

It is a question whether the whole skin is involved in the "psychogalvanic reflex" or only the sweat-glands. The blood vessels have very little to do with the apparent resistance change of the psychogalvanic reflex. The blood corpuscles are highly resistant but the blood plasma is of such low resistance that the whole blood has only moderate resistance. It is necessary to have a layer of cells packed so closely that very few ions can pass between them in order to have a

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very highly resistant structure, like the skin. The resistant layer is the Malpighian layer which extends over the surface of the dermis and is reflected inward to form the sweat-glands. According to Ebbecke the general Malpighian layer can be stimulated only by local irritants and not through these sympathetic nerves, so that (by exclusion) the sweat-glands must be the seat of the "psychogalvanic phenomena." It has been shown by Leva that the "psychogalvanic reflex" is dis-

TABLE I

Change in Polarization Capacity and Resistance (C and r) of the Skin of the Fingers While the Ohmic Resistance of the Fingers Remained Constant at 900 Ohms

Time in minutes	C (μF)	r (ohms)
0	0.0348	9,900
1	0.0350	9,900
2	0.0348	9,900
3	0.0348	9,950
4	0.0349	10,000
5	0.0352	10,000
6	0.0345	10,000
7	0.0340	10,000
7.5	stimulus applied	
8	0.0340	10,000
9	0.0355	8,750
10	0.0357	8,850
11	0.0356	8,950
12	0.0356	9,100
13	0.0355	9,150
14	0.0352	9,200
15	0.0352	9,200

tributed in different parts of the skin in a manner similar to the sweat-glands (Figs. 1 and 2).

Gildemeister (1913) demonstrated that the psychogalvanic reflex is due rather to a decrease in the counter electromotive force of polarization than to a decrease in the true "ohmic" resistance of the skin. In a later paper (Gildemeister (1922)) he gave the relation between this counter electromotive force and the corresponding polarization capacity.

In order to measure true ohmic resistances and polarization capaci-

ties, we have built a Wheatstone bridge of equal ratio arms which, we believe, is superior to any bridge previously used for high-frequency electric currents. When a high-frequency electric current is passed

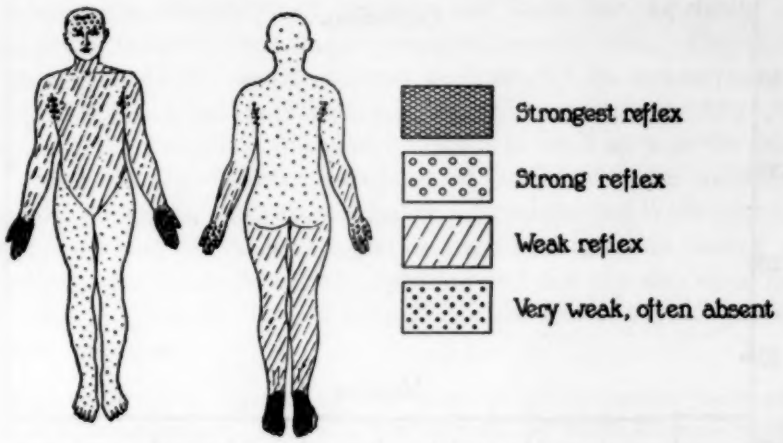

FIG. 1. Distribution of intensity of psychogalvanic reflex according to Leva. The darker portions give the most intense reflex.

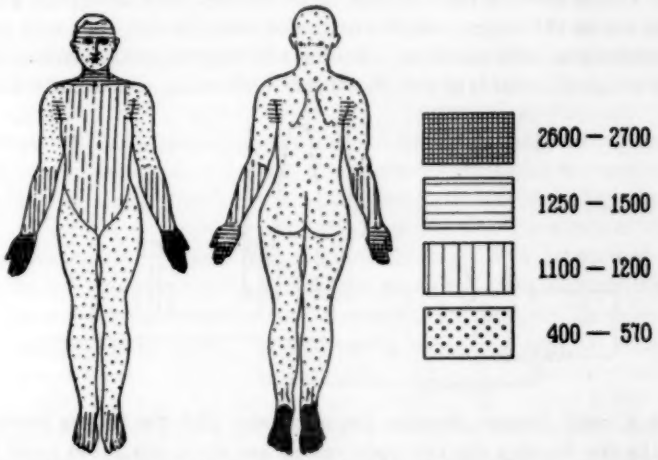

FIG. 2. Distribution of the sweat-glands according to Leva. The darker portions show the most numerous sweat-glands.

through the cell the plasma membrane offers very little impedance to the current. More than 99 per cent of the impedance is the true ohmic resistance of the cell interior.

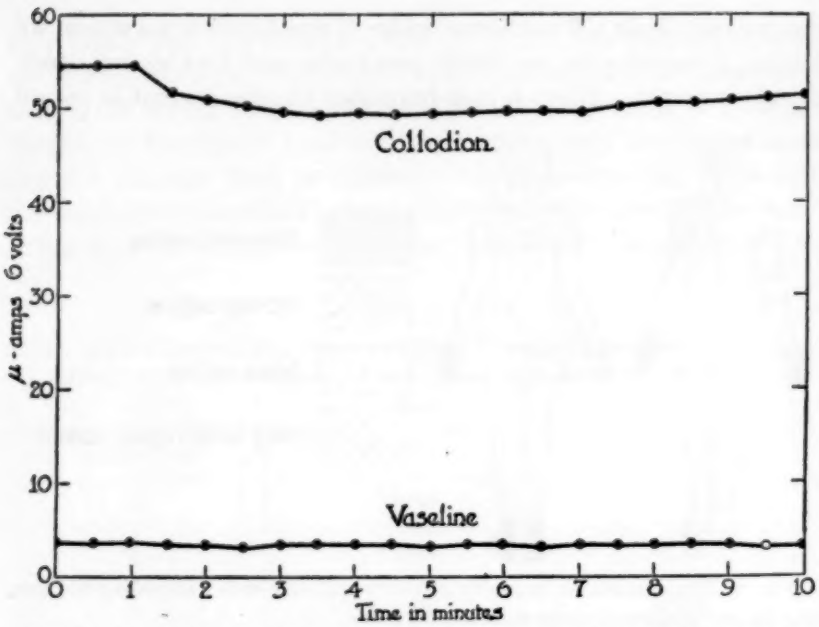

FIG. 3. Graph showing the variation in the current with an applied potential of 6 volts across the fingers, which are coated over in one case with vaseline and in another case with collodion. During a 10 minute period vaseline shows a very high resistance, that is to say, allows only 4 micro-amperes of current to pass.

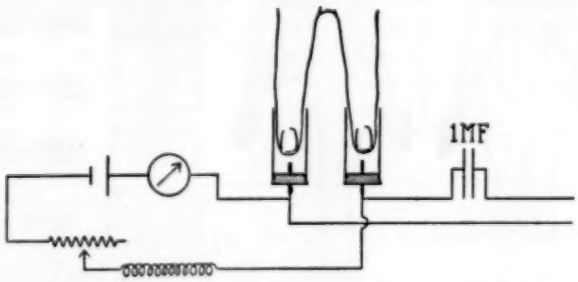

FIG. 4. Circuit diagram showing the electrodes with the fingers immersed in them. (In the drawing the electrode vessels are made much too small for the fingers.) The electrodes are connected on the one end to a direct current circuit, including a galvanometer (indicated by the circle with the arrow) and the choke coil (indicated by the spiral) for preventing a high-frequency current from entering the direct current circuit. The electrodes are also connected to the high-frequency Wheatstone bridge operated with a high-frequency current through a 1 microfarad condenser to prevent the direct current from entering the high-frequency circuit.

The marking off of a constant area of the skin had been done by Densham and Wells by painting with collodion. Our results show (see Fig. 3) that this is a very poor method and probably accounts for the erroneous conclusions of Densham and Wells that the change in impedance is due to stretching following vaso-constriction. Collodion is permeable to the electric current, as shown by the measurements plotted in Fig. 3, and its resistance is changed by stretching. Stretching not only changes its thickness but tends to lift it up from the skin or cracks it. The addition of castor oil (added to make collodion pliable) does not abolish these changes. Densham and Wells assume that stretching the skin changes its resistance but this change is evidently due to stretching the collodion and not the skin since we found that if vaseline is used to mark out the area of the skin, these effects disappear.

Experiments.—Two fingers of the same hand, except the terminal joints, are coated with vaseline and immersed in separate electrode vessels, shown in Fig. 4. The 1 per cent NaCl solution in the electrode vessels is kept at approximately constant temperature. The electrode vessels are connected with the bridge. The arrangement shown in Fig. 4 was used for simultaneous measurements of the apparent direct-current-resistance and the resistance to an alternating current of a million cycles. The average of the results shows that when the apparent resistance to direct current changed 13.7 per cent during the reflex, the resistance to a million cycle current changed 0.93 per cent. For practical purposes then, we may assume that the high-frequency resistance does not change during the reflex. The D.C. apparatus is then removed, the electrodes remaining connected to the bridge and measurements made in quick succession by means of the high-frequency current and then by a current of 1000 cycles per second. By means of the high-frequency current the "ohmic" resistance of the body R is determined and its value in the balancing arm of the bridge then fixed, and after substituting a 1000 cycle current a resistance r and parallel capacity C are connected in series with R and the bridge balanced again. In one case for example, R was 900 ohms and C and r remained almost constant for 8 minutes. After 7½ minutes a stimulus was applied to the other hand by means of an induction coil. After a half minute latent period there was a very sudden drop in r from 10,000 ohms to 8800 ohms. At the same time there was an apparent increase in C from about 0.035 microfarad (μF) to 0.0357 microfarad.

Repetitions of these experiments on a number of persons gave similar results.

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FORWARD MOVEMENT OF PARAMECIUM AS A FUNCTION OF THE HYDROGEN ION CONCENTRATION

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(Accepted for publication, April 9, 1930)

I

An animal highly mobile and possessing the great sensitivity of *Paramecium* under various environmental conditions, should prove useful in quantitative studies on the rate of forward movement, yet Glaser's experiments (1924) describing the effects of temperature on speed of translation appear to be the only ones of this nature. He found that the temperature of the medium influences the rate of swimming, and hence of the reactions upon which this process depends, in the same general way in which it affects ordinary chemical reactions. Hydrogen ion concentration also has a marked effect upon many purely chemical processes. Numerous experiments have been recorded in which *Paramecium* has been treated with acids and bases, but in none of these was the rate of forward movement used as an indicator. Rather, the effects noted were the time of survival in the experimental medium (Dale, 1913; Collett, 1919), or the apparent rate of penetration of the added substance (Harvey, 1911). However, studies in which the rate of locomotion reflects the effects of the added substances are easily carried out.

II

The apparatus employed in these experiments was similar to that used by Glaser (1924). Except in certain specified cases the temperature was kept constant at 24°C. by means of a water bath in which were immersed small U-shaped tubes containing samples of the cultures. Observations were made through a binocular microscope, one of whose oculars contained a micrometer scale. The length of time required by an animal to swim unit distance could then be recorded by means of a stop-watch.

Observations were made in sets of ten. In certain experiments in which the conditions, either within the animal or without, did not remain constant, only one set of observations could be made. In most cases, however, thirty readings constitute an experimental point, and in some instances as many as one hundred and fifty. The readings for any point were averaged and the mean taken as representing the speed of the group of animals as a whole. The objection might be raised that such a method perhaps masks the true state of affairs, since abnormally fast or abnormally slow animals are averaged with the rest and might distort the final mean value. However, by plotting the performance of each individual animal in a certain typical experiment and comparing the mode for each group with the mean calculated for the same group, it was found that no perceptible error arises from this method of recording the data.

The *Paramecia* used in these experiments were all derived from a single individual obtained from a pure line which had been under observation for a year.

The animals were cultured in timothy hay infusion, made by boiling 2.5 gm. of sterile timothy hay (heads and upper stems only) in 250 cc. of water distilled from glass, filtering, correcting for evaporation, and adding 25 cc. of Ringer's solution. The medium was then put into test tubes of about 80 cc. capacity and the *Paramecia* and bacteria added from an already existing culture. The test tubes were stoppered with cotton.

Except in one case experiments were always performed upon animals in culture media of the same age and having the same natural pH.

pH determinations were made by means of the modified colorimetric method suggested by Felton (1921), making possible discriminations to 0.05 pH. It was impractical in most of the experiments to use an electrometric method because the quantities of fluid available for the determinations were extremely small and a micro-electrode such as that used by Bodine and Fink (1925) would have permitted the carbon dioxide, present in considerable quantities in some of the experiments, to escape from the solution while the measurements were being made. The time required for a determination by means of the drop method, on the other hand, amounted to only a few seconds, since the medium was added directly to the indicator. The fact that the drop of medium was handled by means of a fine pipette offering very little liquid surface to the air prevented the escape of more than a negligible amount of CO_2. A salt error hardly enters in these determinations because of the minute quantities of salt present, whereas a protein error is encountered only with very old culture media and when present requires the use of the electrometric method.

Whenever it was necessary to alter the pH experimentally very small amounts of dilute acid or alkali were added to the culture medium in the U-tube. The amount of added substance was not sufficient to cause significant osmotic effects, as was proven by the addition of equal volumes of distilled water which did not alter the speed of swimming. By adding the acid or alkali directly to the culture medium in which the animals were growing disturbance was reduced to a minimum.

III

Bodine (1921) observed in a hay infusion culture containing bacteria and *Paramecia* certain changes of pH correlated with age. Our repetition of Bodine's experiments yielded results essentially similar to his (Fig. 1), although the rate at which the pH changes in the cultures now under consideration is much faster than in Bodine's experiments. As shown in the figure, the rate of change of pH is related to the volume of the medium; more especially, to the ratio of volume to surface.

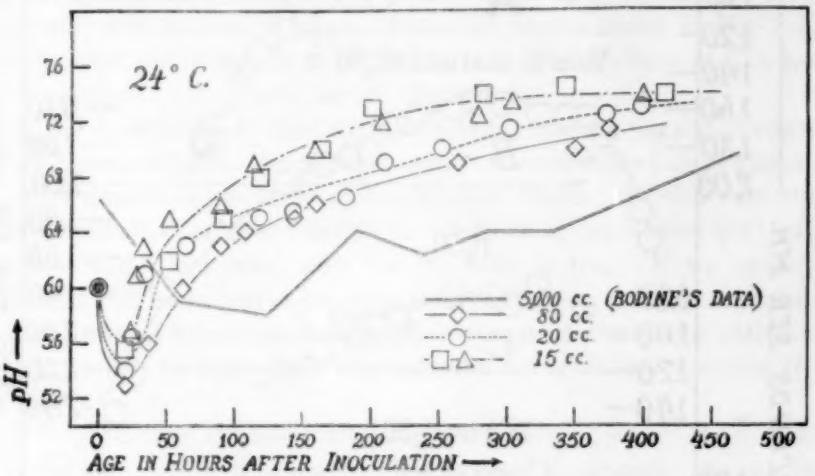

FIG. 1. Natural change in hydrogen ion concentration of different volumes of hay infusion medium, following inoculation with *Paramecia* and bacteria.

While the mechanism of this change of pH falls outside the scope of the present inquiry, the fact itself is well established and makes it possible to observe the effects of pH on rate of forward movement in *Paramecia* without in any way tampering with the medium in which they are living. The results of such observations are shown in Fig. 2 *A*, in which rate of locomotion is greatest when the culture is most acid and decreases markedly as the culture becomes more alkaline.

IV

Is the decrease in speed which occurs as a culture ages and the pH rises, due to the change in hydrogen ion concentration or to an increase

of waste products or possibly to the disappearance of some materials essential to the *Paramecia*? To settle this point the best method is

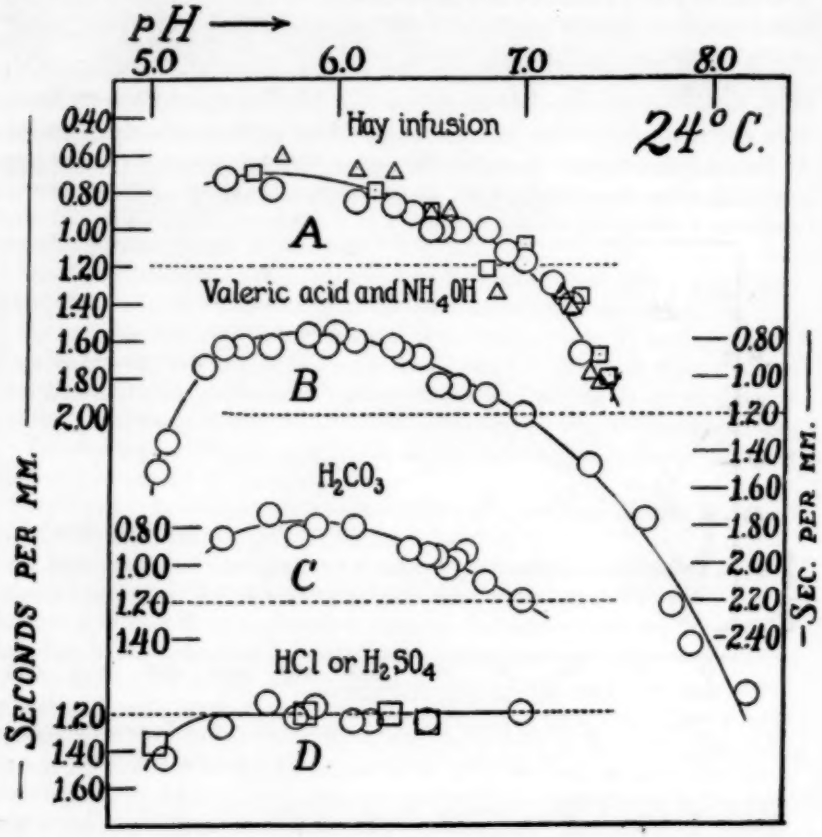

FIG. 2. Rate of forward movement of *Paramecium* following prolonged exposure to different hydrogen ion concentrations. *A*. Naturally-changing hydrogen ion concentration occurring in an aging culture medium. *B*. Hydrogen ion concentrations produced by the addition of valeric acid or ammonium hydroxide to the culture medium. *C*. Hydrogen ion concentrations produced in identical culture media by the addition of carbon dioxide. *D*. Hydrogen ion concentrations produced by the addition of HCl or H_2SO_4 to the culture media.

to alter the pH of samples of a naturally neutral medium (pH 7.0) by artificial means and to observe the effects on speed. Carbonic acid was chosen since this is probably the one most effective in the

natural culture. Two methods were employed. For the range between pH 6.4 and 6.8, CO_2-saturated distilled water was added in quantities not great enough to cause osmotic effects. For the range between pH 5.4 and 6.4 the desired hydrogen ion concentrations were produced by introducing a bit of solid CO_2 into one of the uprights of the U-tube, stoppering, and thus forcing the gas to bubble through the medium. Three or four hours later when the temperature had returned to its original level the rate of locomotion was measured. This speed should correspond with that exhibited by the animals in an equally acid natural medium. From the results shown in Fig. 2 *C*, it is apparent that a given pH produced by the addition of CO_2 has this expected effect upon rate of locomotion.

The duplication of the Curve *A* in Fig. 2 does not yet demonstrate the hydrogen ion as the effective agent. Conceivably CO_2 as such in small quantities might have accelerative effects. In this case the observed effect on speed should be incapable of duplication with any other acid. However, quite the opposite is true. If we employ valeric acid and observe the rate of locomotion at various concentrations, after 3 hours, the data duplicate those obtained with carbonic acid as well as those from the naturally aging culture medium (*B*, Fig. 2).

The hydrogen ion concentrations on the alkaline side of the neutral point were produced by means of ammonium hydroxide. As can be seen from the figure the speed of swimming exhibited by the animals on the alkaline side of neutrality is approximately the same as that shown by animals in a naturally aging culture medium of corresponding pH (*A*, *B*, *C*, Fig. 2). It is unlikely, therefore, that the effect on speed is due to CO_2 itself, but rather to the hydrogen ion dissociated from H_2CO_3.

V

Since carbonic and valeric acids are known to penetrate cells easily, it is altogether possible that the observed effects are due to changes which these acids bring about within the cell. If true, the hydrogen ion is effective not because it is present in the medium, but because it is present in the cell. Accordingly animals exposed for equal lengths of time to media whose pH has been altered by means of inorganic

acids which do not penetrate easily, should not exhibit these changes in speed. To test this, samples of naturally neutral culture medium were adjusted to various pH values by the addition of hydrochloric or sulfuric acids. Observations after 4 hours disclosed a speed of swimming identical with that to be observed in a culture medium of pH 7.0. In other words, although the pH in the medium was made to cover the range from 5.0 to 6.8, the animals behaved as though always exposed to neutrality (*D*, Fig. 2).

The interpretation of this result in terms of permeability differences is rendered more secure by the use of indicators. *Paramecium* readily absorbs methyl red. If such stained animals are introduced into water distilled from glass and rendered slightly acid by means of valeric acid, it is found that after about 5 minutes immersion in the solution many very minute bodies scattered throughout the cytoplasm exhibit a reddish color instead of the uniform yellow which methyl red shows in neutral and slightly acid reaction. On the other hand, when the solution to which the stained animals are subjected is very dilute sulfuric acid this color change does not occur unless the sulfuric acid is sufficiently concentrated to kill. Unfortunately methyl red undergoes its color change at a pH very near that which is fatal to *Paramecium*, so that even in the case of the valeric acid solutions it is necessary to use quantities likely to prove lethal in little more than an hour. However, the fact that a noticeable change occurred soon after immersion in the valeric, but not until movement had ceased in the case of the sulfuric, indicates that valeric acid is able to bring about a decreased pH of some constituents within the unharmed cell.

Experiments in which neutral red was the indicator gave somewhat similar results but these observations are not so reliable because this indicator undergoes its color change in the region of the neutral point (pH 7.0).

The results, as far as they go, agree with those obtained by Chambers with methyl red on *Ameba* (1928), save that he confined his attention to carbonic acid.

From these experiments it is reasonable to assume that within physiological limits the valeric acid, in time, brings about an increase of hydrogen ion concentration in some parts of the interior of the cell, while sulfuric acid is unable to do this. Thus, the changes in rate of

locomotion after prolonged exposure to any given pH may be attributed to changes in the internal hydrogen ion concentration.

VI

Before attempting a theoretical explanation of this result, it is necessary to consider certain immediate increases in speed brought

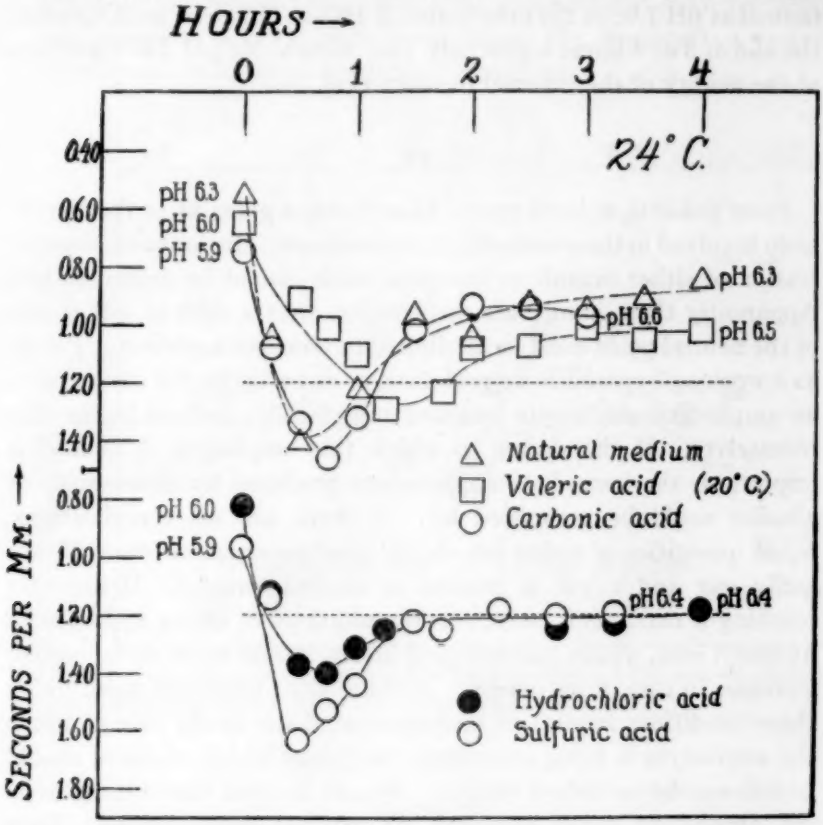

FIG. 3. Variations in rate of forward movement of *Paramecium* during the 4 hour interval following a sudden alteration in the hydrogen ion concentration of the culture medium by various means.

about by the addition of either organic or inorganic acids or alkalies. Short in duration, these increases are followed by rapid declines until after about $\frac{1}{2}$ or $\frac{3}{4}$ of an hour the animals are swimming at rates

rather less than those characteristic in infusions of pH 7.0. Having reached this minimum there follows a period of recovery which lasts about an hour. Finally a speed equilibrium is attained which may be either the normal speed of pH 7.0 or greater, depending on the type of acid to which the animals have been exposed. If the acid be valeric or carbonic, the rate of locomotion after 3 or 4 hours is greater than normal at pH 7.0; on the other hand, if HCl or H_2SO_4 be used, speed at the end of 3 or 4 hours is precisely that normal for pH 7.0 regardless of the acidity of the external medium (Fig. 3).

VII

From the data at hand we can hazard only a guess as to the mechanism involved in these immediate accelerations. Instantaneous penetration of either organic or inorganic acids cannot be demonstrated. Apparently then, immediate acceleration after a shift to either side of the neutral point must be attributed to some surface effect. Purely as a working hypothesis we postulate, as essential for the ciliary beat, an amphoteric electrolyte localized superficially, perhaps in the cilia themselves. If the degree to which this ampholyte is ionized is important, the immediate accelerations produced by either acids or alkalies could be accounted for. If there are no complications, equal quantities of either ion should produce equal effects. Necessarily our ampholyte is present in limited amount. Hence after reaching a maximum speed, further additions of either hydrogen or hydroxyl ions, within physiological limits, should result in no further increases in rate of movement. Finally, since increased speed under these conditions implies an uncompensated rise in the rate at which the ampholyte is being consumed, the initial bursts of speed should be followed by periods of decline. We can imagine that during these, the ampholyte could approach its original concentration. With inorganic acids therefore we should find a restoration of the speed characteristic in the original medium; with organic acids however, the final speed should be perceptibly greater. The higher speed equilibrium manifest after 3 or 4 hours of continuous exposure to valeric or carbonic acid, in terms of this hypothesis, must be attributed to the ultimate and demonstrable penetration of the hydrogen ion. This

internal rise in [H′], we assume, is followed by an actual acceleration in the rate at which the essential ampholyte is synthetized. Accordingly, immediate and final increases in speed of swimming involve essentially the same mechanism. Under the conditions of these experiments increased ionization of a constant quantity of ampholyte would be indistinguishable from an increase in the total amount available for dissociation. While the experimental evidence behind this reasoning is incomplete, nevertheless Fig. 4 suggests a very fair approximation to the theoretical requirements.

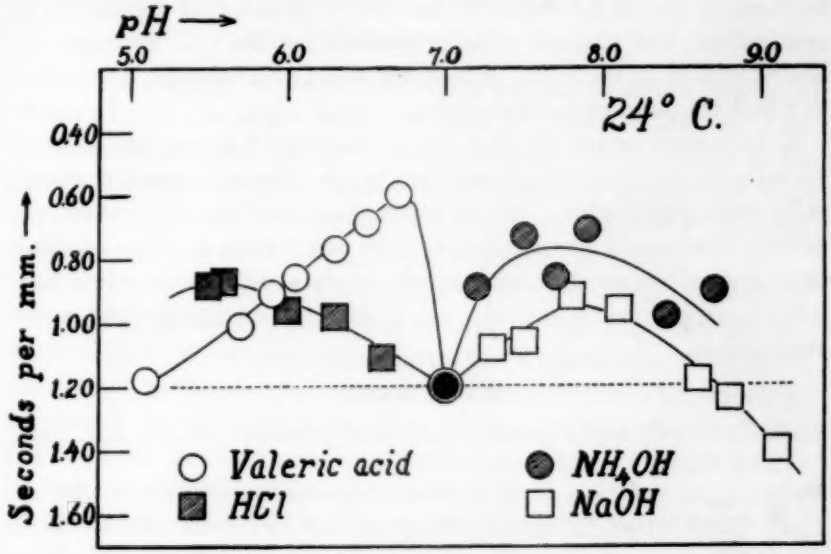

FIG. 4. Immediate effect on rate of forward movement of *Paramecium* of alterations in hydrogen ion concentration of the culture medium.

VIII

SUMMARY

1. At constant temperatures, and within physiological limits, changes of pH in either direction from the neutral point result in immediate increases in speed of movement of *Paramecium*.

2. These increases are temporary. In 30 to 45 minutes a minimum of speed is reached. This is followed by a period of recovery lasting about an hour. Finally an equilibrium is found. With inorganic

acids (HCl or H_2SO_4) the final speed after 3 or 4 hours is that characteristic of prolonged exposure to pH 7.0; on the other hand, 3 or 4 hours after the application of either valeric or carbonic acid, speed is proportional to the [H′] of the external and, probably, of the internal medium.

3. These facts become explicable if we assume that the ionization of an ampholyte superficially localized is essential for the execution of the ciliary stroke. Valeric and carbonic acid, in time, demonstrably penetrate the cell. As a working hypothesis we postulate that internal increase of the [H′] accelerates the rate at which this ampholyte is synthetized; but without actually penetrating the cell, hydrogen or hydroxyl ions in the external medium could also increase the degree to which this ampholyte dissociates.

4. Increased ionization of a fixed quantity of ampholyte and an increase in the rate of its production are in these experiments practically indistinguishable. Hence we assume that immediate and temporary increases of speed resulting from any change of pH, as well as final and permanently higher speed levels manifest only after prolonged exposures to organic acids, involve essentially the same mechanism.

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HEATING OF SIMPLE SOLUTIONS AND EMULSIONS EXPOSED TO HIGH FREQUENCY HIGH POTENTIAL ELECTROSTATIC FIELDS

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INTRODUCTION

The first experiments recorded in the literature on the physiological effects of high frequency fields are those of Gosset, Gutmann, Lakhovsky, and Magrou (1924).[1] They found that plant tumors in the geranium caused by innoculation with *Bacterium tumefaciens* were successfully treated by exposure to the field of a 2 meter oscillator. However, the details of the method of exposure are lacking.

In 1926 J. W. Schereschewsky published a report on the temperature rise and killing times in mice when exposed to high frequency electrostatic fields.[2] From these experiments he concluded that the 6 meter wave would be the most valuable for further experiments on pathological tissue. At this point it should be emphasized that this conclusion was based on data taken at low potential gradients. Two years later Dr. Schereschewsky published data[3] on experiments with a strain of mouse sarcoma known as the Crocker Research Laboratory's No. 180 and the Rous fowl sarcoma. He used a 6 meter wave and a high potential gradient across the affected tissue.* The results were very encouraging and led other investigators into the field.

Christie and Loomis,[4] in an investigation of the heating effects in mice placed in fields of the same order of intensity as those used in Schereschewsky's first experiments, showed that the rate of rise of temperature in a M/20 saline solution was practically the same in the body of a live or dead mouse for wave-lengths down to 6 meters, but that from 6 to 2 meters the tissue heated less rapidly than the saline

* This latter I infer from the experimental arrangement.

solution and that the killing time was roughly proportional to the rate of rise of the temperature. They also made the first rate-of-temperature-rise wave-length curve. However, they used constant current through the condenser instead of constant voltage on the plates of the condenser.

An investigation into the effect of high frequency fields on *Paramecium caudatum* was reported in 1929 by Kahler, Chakley and Voegtlin.[5] Their data indicated that for both magnetic and electrostatic fields the lethal effect was roughly proportional to the rise in temperature whether the solution was heated by high frequency fields or hot water bath. The voltage across the condenser was not measured but in both cases (magnetic or electrostatic fields) the field intensities were evidently of low value.

PRELIMINARY WORK

During the summer of 1929 the author, while a student of Dr. Hoag at the University of Chicago, made some preliminary observations with apparatus similar to that used by Christie and Loomis. However, in addition to measuring the current through the condenser the voltage on one of the plates was measured with an electrostatic voltmeter. The potential gradients were of low value (about the same order as used on the mice). The ordinary equation connecting power loss (P) with voltage (V) and current (I) (*i.e.*, $P = KVI$) was applied. P was taken as the rate of temperature rise. V was the value of voltage found by observing the 60 cycle voltage necessary to produce the same deflection of the electroscope. I was the current as measured by a thermocouple and microammeter calibrated at a frequency of 7.5×10^6 cycles per second (40 m.), and connected in the circuit 3 or 4 cm. below the lower condenser plate. The curve obtained is shown in Fig. 1.

The maximum voltage obtainable at 3 meters was only 60 volts, so, taking into account the spacing of the condenser plates and the thickness of the glass in the cell, the potential gradient in the solution could not have been more than 30 volts per centimeter. The current measurement at the lower wave-lengths was in error due to skin effect in the thermocouple. For instance, using the standard A.C. resistance formulae[6] and calculating the current necessary to produce the same

temperature in a No. 40 manganin wire at a frequency of 5×10^7 and 10×10^7 respectively, it was found that at the higher frequency a current of 0.9 ampere should show the same deflection as 1 ampere at the lower frequency. However, this correction would not account for a significant part of the decrease in the value of K below the frequency of approximately 5×10^5 cycles.

This decrease in absorption (rate of temperature rise) below 6 meters is hard to reconcile with the theoretical and experimental work on anomalous dispersion and absorption at radio frequencies that has been advanced by Debye and others.[8] Such considerations indicate that in general the absorption should increase for shorter wave-

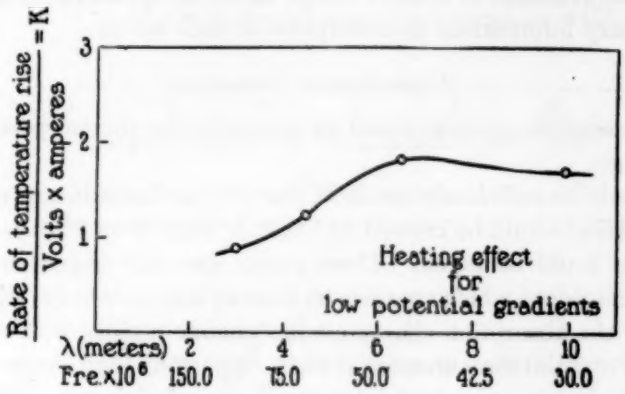

FIG. 1

lengths rather than decrease as shown in Fig. 1. Hence, in view of the fact that low potentials only were used to obtain this curve, it became desirable to find out whether the same relations held for high potential gradients.

For the problem of the physiological effect of high frequency fields there are two general possibilities to be considered. One is a specific action on the chemical complex and the other one is a secondary effect due to the production of heat. The latter is unavoidably present and might conceivably mask the former factor. There is also the possibility of differential dissipation of energy (such as heat) due to the chemical and structural characteristics of the cells and tissue. In his second paper, Schereschewsky remarks that microscopic examination

of the treated tissue revealed general necrosis, the tumor cells being affected most with their nuclei "bearing the brunt of the attack." However, the tissue was exposed to field intensities incomparably greater than those used in all reported work on the thermal effects in normal tissue, as just reviewed above. It is obvious that any work toward the solution of the problem should involve field intensities (potential gradients) of the same order as those that have proven successful in treatment, and that, as a matter of practical procedure, the thermal effects as a function of potential gradient, frequency, specific chemical constitution and structure (cellular structure and colloidal structure) should be investigated first. Accordingly, when the author returned to Beloit College, he set up apparatus to get some preliminary information on absorption in such media.

Experimental Procedure

The essential requirements of an apparatus for this purpose are the following:

It should be sufficiently sensitive that only an insignificant temperature interval would be crossed and that at least 10 to 20 observations per hour could be taken. These points are very important in the case of emulsions which separate on heating and are always significant because the absorption characteristics change rapidly with temperature. Potential measurement is more important than current measurement because the field intensity is the vital factor. To obtain high potential gradients and also to fulfill the above requirements, only a small amount of material should be necessary.

These requirements were partially satisfied by a modification of a differential air thermometer described by Owens.[7] This was arranged to give the relative rates of rise of temperature of liquids contained in a small mica cell placed in an intense electrostatic field. The voltage on one of the condenser plates was measured by means of an electroscope. An auxiliary heating device operated by 60 cycle current was used to standardize all rates of rise of temperature measurements. The latter were obtained by taking the time necessary for the light beam to move a specific distance on the scale as measured with a stop watch. The instrument was carefully checked and found to have a straight line characteristic and to give fairly consistent readings. The condenser was connected in an auxiliary circuit which was tuned to a "driver" circuit. The latter was operated with a D.C. plate supply. It is to be noted that this differs not only from the experimental work

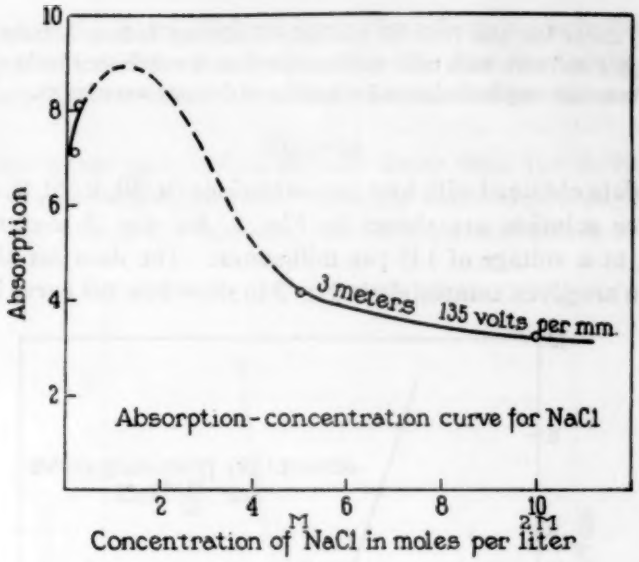
Absorption
10
8
6
4
2
3 meters
135 volts per mm.
Absorption-concentration curve for NaCl
2
4
6
8
10
M
2 M
Concentration of NaCl in moles per liter

FIG. 2

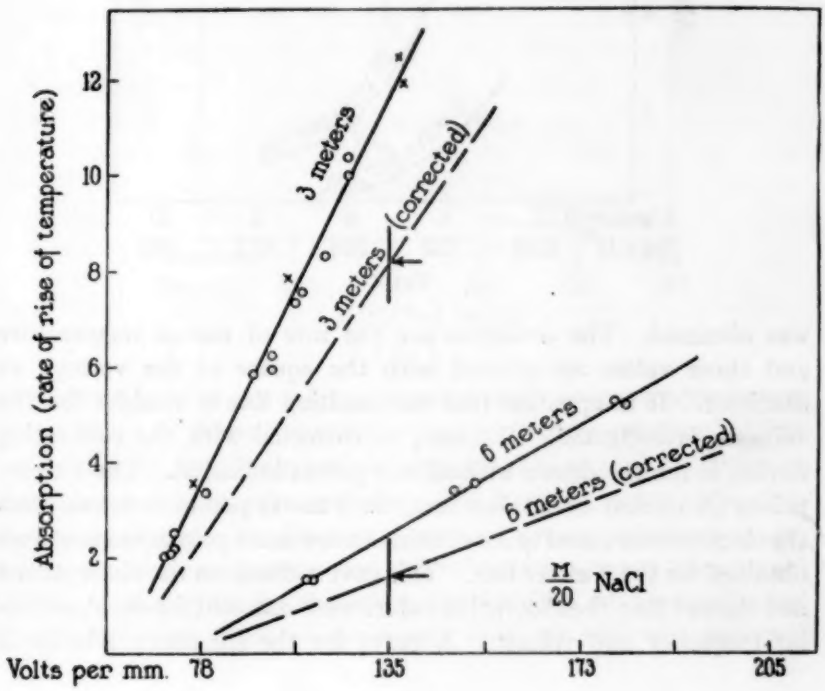
Absorption (rate of rise of temperature)
12
10
8
6
4
2
3 meters
3 meters (corrected)
6 meters
6 meters (corrected)
M/20 NaCl
Volts per mm.
78
135
173
205

FIG. 3

reviewed above but also from all available diathermy technic because the D.C. plate supply and a vacuum tube oscillator produce a continuous train of oscillations of constant amplitude instead of a series of damped wave trains.

RESULTS

The data obtained with four concentrations (M/40, M/20, M, and 2M) of saline solution are shown in Fig. 2, for the 3 meter wavelength, at a voltage of 135 per millimeter. The data for the M/20 solution are given completely in Fig. 3 to show how the curve in Fig. 2

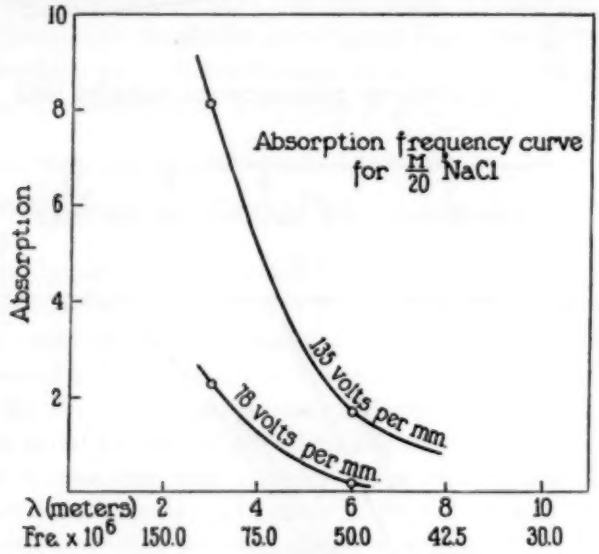

FIG. 4

was obtained. The ordinates are the rate of rise of temperature and these values are plotted with the square of the voltage as abscissae. It is apparent that the resulting line is straight for the voltages investigated. The line, as corrected with the calibrating device, is the one drawn without any points indicated. The 3 meter points (in circles) were taken first, the 6 meter points next, and then the circuits were tuned to 3 meters and a few more points (crosses) were obtained for the 3 meter line. This gave a check on the observations and showed that the absorption values were constant for each particular frequency and voltage. A curve for the frequency relation is

shown in Fig. 4. Some work on saturation effects in electrolytes has been reported[8] and further work along this line may yield useful physiological information, as well as information on the nature of solution.

Distilled water gave values slightly lower than the M/40 NaCl solution, and as observations were made, the values were always found to be falling. From the data obtained, three series of observations on

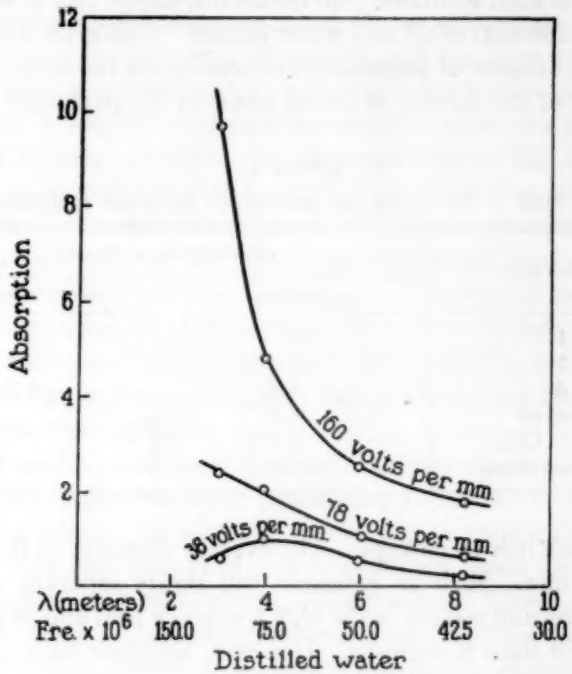

FIG. 5

each of two samples, it was found that the values always dropped about 40 per cent and thereafter remained approximately constant. At no time, however, were observations obtained that were as consistent as those found for the saline solutions, the latter of which showed no drop on exposure. The frequency curve for several voltages is shown in Fig. 5. It is noteworthy that this information, taken from graphs similar to that given for the saline solution (Fig. 4), agrees qualitatively with that obtained previously (Fig. 1) with low potential

gradients (2 to 4 volts per millimeter) if the first exposures only are considered.

The case of the emulsion is the most significant for the physiological problem. Cottonseed oil was adjusted to the same density as the 1 per cent sodium oleate solution by the addition of approximately one part of the carbon tetrachloride to five parts of cottonseed oil.* The solution was added to the sodium oleate solution in small portions with shaking after each addition. All emulsions, except No. 3, were made with equal amounts of oil and water phases. This gives an excellent emulsion; its degree of permanence depending on the accuracy of the adjustment of the density of the oil phase at the particular tempera-

TABLE I

3M (100 × 10^6 cycles per second), 67 volts per millimeter

Emulsion No.	Relative values of absorption	
	1st run	2nd run (24 hours later)
1	14	3
1	13	3.2
3	8.3	8.3
Cottonseed oil	1.7	
" CCl_4	1.9	
1% Sodium oleate	3.1	3

ture at which it is to be kept. The average diameter of the droplets was 0.056 mm. Such an emulsion will slowly separate under the slight heating and cooling in the high frequency field and so successive readings will show a decrease. However, sufficient data can be obtained before this factor becomes serious.

Determinations were made on the cottonseed oil and the cottonseed oil carbon tetrachloride solution, the 1 per cent sodium oleate solution, and the emulsions. A great difference between the values of the components and the emulsions was found in every case (see Table I). In view of the high values obtained for the emulsions, the high values for the 1 per cent sodium oleate may be said to be due to its colloidal state.

* The author is indebted to Mr. E. W. Toepfer for suggesting this emulsion.

The emulsions slowly separated under exposure and it is significant that the drop in energy loss is much greater than that found for water. For instance, the first exposures at 3 meters on one sample dropped from 14 units to 3.0 units, another from 13 to 3.2. In both cases the second observations were taken 24 hours after the first. The state of the emulsions cannot be accurately compared but it would appear that this demonstrates the importance of the colloidal state. The emulsions never completely coagulated, the upper layer always became a "thin" emulsion of water in oil and similarly the lower layer always remained oil in water with the oil-water ratio much decreased.

Emulsion 3, was the only emulsion used in which the amounts of water and oil were not equal. In the preparation of this sample, too much carbon tetrachloride was added to the oil, so that, on standing, the oil settled. However, it remained in the disperse state and the final result was that most of the water (approximately $\frac{1}{3}$) was removed from the spaces between the oil droplets. The rate of temperature rise in this emulsion was considerably lower than the two samples of Emulsion 1, but the coagulation effect due to settling out could not proceed to a degree comparable with that of Emulsion 1 and the drop of absorption was negligible.

While these data indicate a significant increase of loss in the case of colloids, these results are merely preliminary and should be established for other emulsions. However, it has long been known that impure dielectrics give high values of power factor on much longer wavelengths, as measured with bridge arrangements. These data show that at the potential gradients Schereschewsky used on pathological tissue, the higher values of frequency should be investigated for physiological effects. In the case of living tissue it is evident that differential dissipation of energy (as heat) in the cell and between cells of different characteristics is to be expected on a basis of specific chemical composition and colloidal organization as well as cellular structure.

SUMMARY

1. It is shown that the absorption in liquid dielectrics is a function of potential gradient (field intensity) as well as frequency and that for values of potential gradient above, at least 70 volts per millimeter,

the rate of rise of temperature-frequency curve increases rapidly with frequency.

2. The presence of ions in measurable quantity considerably changes the absorption characteristics and apparently causes the values to remain constant, whereas the values for water drop about 40 per cent, during exposure. The absorption also changes rapidly with the concentration of the electrolyte.

3. Very high absorption values are found for an emulsion of cottonseed oil in 1 per cent sodium oleate. It is shown that the absorption is due to the colloidal structure (with the possibility that the energy is dissipated at the phase boundaries).

In conclusion the author takes great pleasure in thanking Professor V. A. Suydam of Beloit College and Dr. J. Barton Hoag of the University of Chicago for their continued advice and encouragement during the work.

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THE GEOTROPIC CONDUCT OF YOUNG GUINEA PIGS

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(Accepted for publication, April 11, 1930)

I

The possibility of obtaining quantitative description of geotropic orientation as related to the magnitude of the effective gravitational excitation has been demonstrated for several animals such as young rats (Crozier and Pincus, 1926–27; Pincus, 1927; Pincus and Crozier, 1929), mice (Crozier and Oxnard, 1927; Keeler, 1927–28), slugs (Wolf, 1927; Wolf and Crozier, 1927–28), arthropods (Crozier and Stier, 1928; Kropp and Crozier, 1928; Kropp, 1929; Crozier and Stier, 1929), and chicks (Hoagland, 1929). The interpretation of the observed facts of geotropic behavior leads to the view that orientation upon an inclined surface is determined by the distribution of the muscular stresses resulting from the pull of the animal's weight upon the two sides of the animal's body. It is assumed that an orienting animal orients until the stress or pull upon the two sides of the body is in a sensory way equivalent.

It has been found that young rats and mice orient in a way such that the relation between the angle of orientation θ and log sin α, where α is the angle of inclination of the plane upon which the animal creeps, is slightly sigmoid. These animals creep with their bodies in contact with the substratum. Because of this characteristic it was desired to investigate the geotropic conduct of a mammal which progressed with the body clear of the supporting surface.

II

Young guinea pigs (age 4 to 14 days) were chosen, and the technique employed in the collection of the data was essentially the same as that used in the work with the rats and mice.

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* National Research Council Fellow.

The plane used in this experiment was approximately 3 feet wide and 4 feet long, being hinged at one edge so that its inclination could be varied. The angle of inclination was measured with a protractor and plumb line fastened to one edge of the plane. The plane was covered with a fine meshed wire grid which afforded the animals a good foothold for progression over the surface of the plane. The animals were placed upon the plane with the body axis horizontal. From this position the animal nearly always moved forward and upward until oriented, and then progressed rapidly at a relatively constant angle with relation to the base of the plane until the edge of the plane interfered with further progression. All observations were made in total darkness and markers of luminous paint attached to the mid-region of the animal's back enabled the observer to follow clearly the animal's movements. The ends of coordinate lines upon the plane were marked with luminous spots which were below the edge of the plane so that they were out of the visual field of the animal. The progression of a guinea pig upon the inclined plane is rapid and even, and relatively long runs (60 to 90 cm.) are generally made without stopping.

The path of an animal upon the plane is clearly observable, and notation of the relation of the path to the luminous spots at the ends of the coordinate lines upon the plane enables the experimenter to make accurate record of the path upon coordinate paper placed above a glass-topped box dimly lighted from below with a red light. The earliest portion of each trail (a few centimeters) was curved, since an animal moves forward and orients simultaneously. In collecting the records the initial curved portion of the path was naturally disregarded and the straight portion of the path recorded, since it is obviously only the straight part which represents complete orientation. Because of the extreme variability of the trails at the very low angles no records were collected for slopes below $\alpha = 15°$. Collection of data for angles of inclination above $\alpha = 60°$ was impossible because the animals cannot then obtain a foothold on the surface of the plane. The entire experiment was carried on at a nearly constant room temperature of 20°C. Two series of observations were made upon animals obtained from the Bussey Institution. Little is known of their genetic constitution except that close inbreeding had been carried on for several generations.

III

Table I presents the extents of upward orientation θ in the cases of eight young guinea pigs with various inclinations α of the plane of progression. Ten trails of each animal were recorded at each inclination. Each value therefore is the mean of eighty observations. Table II presents the mean angles θ of trails of geotropic orientation in the cases of six young guinea pigs with various inclinations α of the plane of progression. Each value of θ is here the mean of sixty observations. The data presented in this table were collected approximately 40 days after those presented in Table I, and the animals used were on the

average about 6 days younger than those used in the earlier experiment.

As in the cases of young rats and mice (Crozier and Pincus, 1928; Crozier and Oxnard, 1927) it is apparent that there exists some func-

TABLE I

Data for Eight Animals of Series I

Each value of θ is the mean of 80 observations

α	θ	P.E.
15	46.9	±1.32
20	47.3	±0.97
25	54.7	±0.86
30	59.4	±0.74
35	61.9	±0.68
40	66.1	±0.62
45	65.7	±0.90
50	77.0	±0.59
55	81.7	±0.45
60	86.5	±0.24

TABLE II

Data for the Six Animals of Series II

Each value of θ is the mean of 60 observations

α	θ	P.E.
15	44.8	±1.18
20	46.2	±0.84
25	51.9	±0.67
30	57.9	±0.46
35	60.6	±0.59
40	66.9	±0.50
45	68.3	±0.58
50	76.1	±0.46
55	81.2	±0.37
60	85.0	±0.32

tional connection between the angle of orientation θ and the inclination of the plane of progression α. And that the relation between θ and log sin α is fundamentally sigmoid in character is definitely illus-

trated for both series of animals in Fig. 1. However, the curves appear to be compound, with a break clearly apparent at $\alpha = 45°$. No obvious reason for the occurrence of this break could be discovered until a blinded animal was tested in a lighted room at the several values of α. In the region of $\alpha = 45°$ the mode of progression changes. Below this value the guinea pig moves forward by walking with the

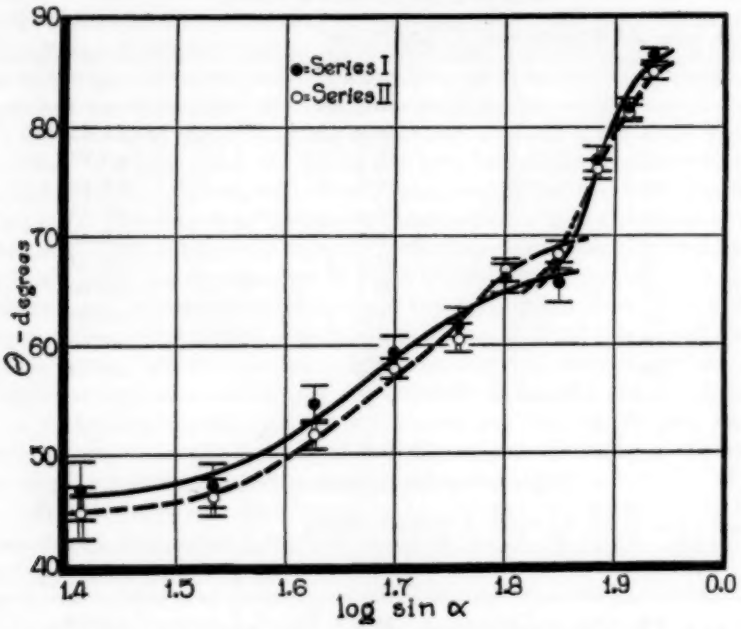

FIG. 1. Angles of orientation for the two groups of animals. The compound character of the relation between θ and α is apparent in this figure. The height of the vertical bars = 2 P.E.

legs on the two sides of the body extended alternately. Above $\alpha = 45°$ progression is by a hopping movement in which both back legs, and alternately both front legs, are extended simultaneously. This furnishes us with a definite basis for the break that occurs in the plotted functions. Inasmuch as there is a totally different muscular coordination involved in the mode of progression which appears at the higher values of α, it is reasonable to assume that a different distribution of muscle tensions would exist in this case, thus bringing about a change in the relation of θ to α.

That we are dealing with strictly comparable phenomena in the cases of the guinea pigs and other young mammals studied (Crozier and Pincus, 1928; Crozier and Oxnard, 1927) is demonstrated in Fig. 2, where cos θ is seen to decrease rectilinearly as sin α increases, but with two regions in which the proportionality factor differs.

To a fair approximation, the relative variation of the mean θ declines rectilinearly with increase of log sin α (Fig. 3), as with young rats and mice (Crozier and Pincus, 1928–29; Crozier and Oxnard, 1927; Crozier,

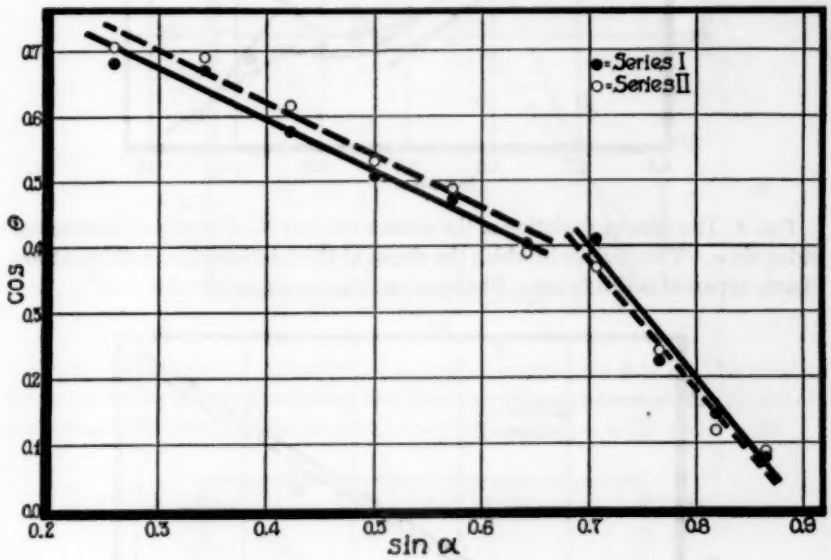

FIG. 2. The cosine of the angle of upward orientation θ is directly proportional to the sine of the inclination α of the plane of progression. The two regions in which the proportionality factor differs are clearly represented in this figure.

1929) but again two regions are clearly evidenced in which the slopes of the relationship are different. It is a noteworthy fact that just as in Fig. 1 where careful inspection discloses a zone of discontinuity at approximately $\alpha = 45°$, so also in the variability plot the discontinuity already referred to makes its appearance at precisely the same point. In another way the reality of the discontinuity can be demonstrated. It is not without interest to observe that if for these measurements we plot log θ against slope α, the relationship is very nearly a straight line

(Fig. 4), with however indication of a cusp at $\alpha = 45°$. If with the same coordinates we plot the log (P.E.$_\theta/\theta$) against α (Fig. 5), the graph

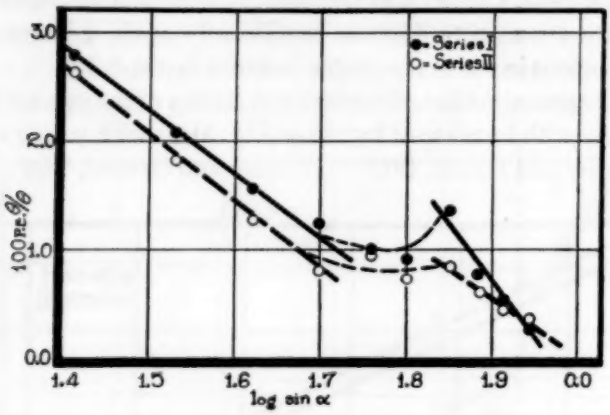

FIG. 3. The relative variation of the mean θ declines rectilinearly with increases of log sin α. Two regions in which the slopes of the relationship are different are clearly apparent in this figure. The break occurs approximately at $\alpha = 45°$.

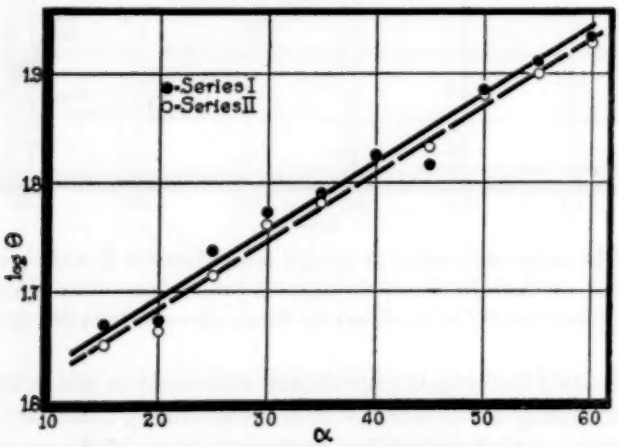

FIG. 4. The relationship here (log θ *vs.* α) is very nearly a straight line. However in the light of information obtained by plotting on the coordinates (see Figs. 1 and 2) a cusp is indicated at $\alpha = 45°$.

is once more composed of two straight lines with a sharp break in the region of $\alpha = 45°$. These facts are equally evident in the two inde-

pendent series of measurements. The interest in this finding comes partly from the fact that it illustrates the empirical character of the

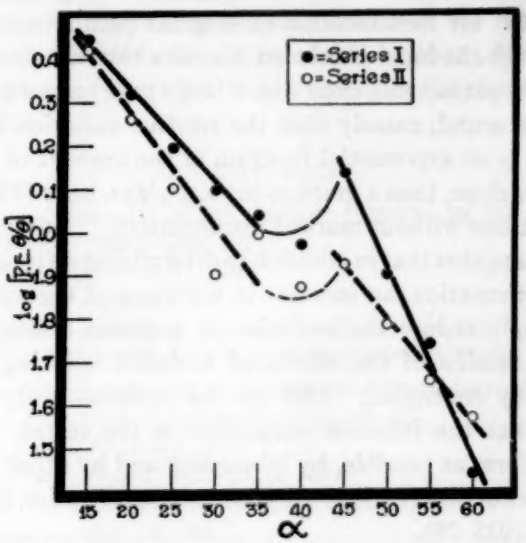

FIG. 5. That there is a critical point located approximately at $\alpha = 45°$ is clearly demonstrated by plotting log (P.E. $_\theta/\theta$) against α. The graph is again composed of two straight lines with an obvious break occurring between $\alpha = 40°$ and $\alpha = 50°$.

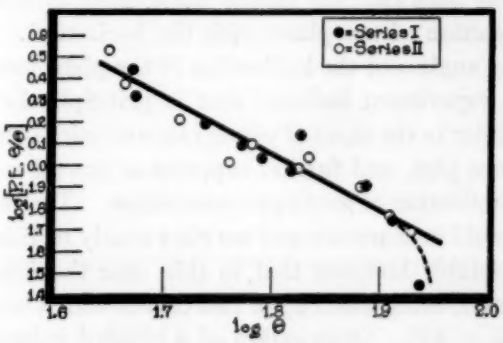

FIG. 6. As can be deduced from Figs. 4 and 5, log [P.E $/\theta$] should be very nearly a straight line function of log θ. Within reasonable limits of precision this is approximately true. The observations of Series II have been multiplied by anti-log 0.09 to facilitate comparison. For comment compare text.

formulations chosen, and in certain cases their unexpected form (compare Fig. 4, log θ *vs.* α); and further from the demonstration of the value of measures of variation computed under proper conditions (Crozier, 1929) for identification of singular points in curves of behavior, through the inner checks on the data thus obtainable. If the considerations extractable from Fig. 4 (log θ *vs.* α) and Fig. 5 (log P.E. $/\theta$ *vs.* α) are sound, namely that the relative variation in geotropic performance is an exponential function of the amount of orientation, with negative slope, then a graph of log P.E. $_\theta/\theta$ *vs.* log θ (Fig. 6) should be a straight line without marked discontinuity. This comes clearly from the assumption that in addition to determining an increased extent of upward orientation, an increase in the slope of the surface should proportionately reduce the variation in response through a proportionate suppression of the effects of variation-inducing factors not experimentally controlled. This can be examined only under such conditions that the inherent variability of the tested organisms is made as uniform as possible, by inbreeding and by other precautions, and if number of observations are kept uniform (Crozier, 1929; Crozier and Pincus, 1928–29).

SUMMARY

Young guinea pigs while progressing on an inclined surface orient upward in a way such that the path of progression is at a mean angle θ to the intersection of the plane with the horizontal. The angle θ increases as the angle α of the inclination of the plane increases. The results of this experiment indicate that in principle the formulation of similar behavior in the cases of young rats and mice holds essentially for young guinea pigs, and further supports a general muscle tension theory of the limitation of geotropic orientation. The relation of θ to log sin α is sigmoid in character and cos θ is a nearly rectilinear function sin α. It is notable however that in this case the functions are in reality compound, being made up of two curves with a break occurring at a slope of $\alpha = 45°$. Observation of a blinded guinea pig in light upon an inclined plane reveals the fact that in the neighborhood of 45° the mode of progression changes. Below this angle the animal walks with the feet on the two sides of the body moving forward alternately, while above $\alpha = 45°$ the animal hops in such a way that both front

feet and both hind feet move forward together. This change in the mode of progression clearly involves a change in the organization of muscular tensions, and in all probability accounts for the change in the relation of the values of θ to the magnitudes of the slope. The behavior of the mean θ's is closely paralleled by that of their P.E.'s, an automatic check being in this way given upon the significance of the measurements.

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ELECTROKINETIC PHENOMENA. II

The Factor of Proportionality for Cataphoretic and Electroendosmotic Mobilities*

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I

INTRODUCTION

According to the Helmholtz-von Smoluchowski (1) theory of cataphoresis, the equation for V_p, the cataphoretic velocity of a particle relative to a given medium is,

$$V_p = \frac{1}{4\pi} \frac{XD\zeta}{\eta} = \frac{CX}{\eta} \tag{1}$$

(X = field strength; D = dielectric constant of the medium; ζ = electrokinetic potential; η = viscosity of the medium; C = constant; all units c.g.s. electrostatic.)

Equation (1) predicts that (1) cataphoretic mobility should be independent of size and shape of the particle, and (2) for similar surfaces (ion atmospheres), V_E, the electroendosmotic velocity of a liquid past the surfaces should be equal to V_p, the velocity of the particle through the liquid.

Debye and Hückel (2) on the other hand, maintained on theoretical grounds that the constant, $\frac{1}{4\pi}$, in Equation (1) was valid only for the cataphoresis of cylindrical particles. For spherical particles the factor, 1/6, was substituted. We have previously shown by experiment in collaboration with Freundlich, and later with Michaelis (3), that the cataphoretic velocity of microscopic particles having similar

* Presented at the Eighth Colloid Symposium, June, 1930.

surfaces is independent of their size and shape. There is, in addition, evidence that as a first approximation this independence of velocity of size may extend to the order of magnitude of the radius of the egg albumin molecule far below the limits of microscopic visibility (4).

While these experiments were performed with rather extreme variations of size and shape, they were not definitely a test of that boundary condition of Debye and Hückel's theory which assumed that the radius of curvature of the cylinder was very large in comparison with the thickness of the ion atmosphere. An experimental investigation including a test of the boundary condition would be the measurement of cataphoretic mobility of particles in a given medium simultaneously with the electroendosmotic mobility of the medium relative to a flat surface[1] having an ion atmosphere identical with that of the particle. Substituting the values of Debye and Hückel for C in Equation (1) and solving for R, the ratio of V_E and V_p, we obtain

$$R = \frac{V_E}{V_p} = 1.5$$

In other words, according to this theory electroendosmotic mobility must be 50 per cent greater than cataphoretic mobility.

II

HISTORICAL

Mooney (5) appears to be the first investigator to attempt to evaluate R. Among Mooney's as yet unexplained findings was the fact previously noted that oil droplets in dilute electrolytes have migration velocities that increase with particle size. Dilute $CuSO_4$ solutions anomalously abolished this effect. In these $CuSO_4$ solutions, therefore, mobility was independent of size. Taking advantage of this fact, Mooney wet the inside of a round capillary tube with a paraffin oil and studied in this system the cataphoretic velocity of the oil droplets and the electroendosmotic velocity of the liquid against a surface presumably covered with oil. In one system V_p was very nearly equal to V_E.

[1] An absolutely flat surface is, of course, not realizable experimentally.

The data of van der Grinten (6), obtained in a flat cataphoresis cell, are in contrast to the finding of Mooney that $R = 1.0$ (approximately) for a round capillary. Van der Grinten studied the cataphoresis in distilled water of small glass particles made of the same glass coverslips from which his flat cataphoresis cell had been assembled. He thus assumed that the surfaces of the particles of glass powder obtained by breaking up his coverslips were the same as that of the flat uninjured coverslip. Van der Grinten interpreted his data to give a mean value of $R = 1.59$, thus apparently confirming fairly well the theory of Debye and Hückel. Abramson (7) powdered pyrex glass and repeated the experiments of van der Grinten with a cell made of the same pyrex glass. This author found that for a given cataphoresis cell, R varied from 1.27 to 3.2 as a function of the nature of the medium. This cell of pyrex glass was not of uniform rectangular cross-section. The values obtained for R were consequently not considered absolute but rather pointing to the fact that a complete reinvestigation of the value of R was necessary under known hydrodynamic conditions and where the flat surface and surface of the particle were chemically identical.

III

The Movement of Liquids in Flat Cataphoresis Cells, Produced by Electroendosmosis

The movement of liquids in flat cataphoresis cells has been previously adequately considered for cells of various types by Ellis (8), von Smoluchowski (1), Svedberg and Andersson (9), Tuorilla (10), Freundlich and Abramson, and Abramson (11). Since the recalculations to be made here of certain data depend upon the movement of liquids in cataphoresis cells, we shall briefly review the facts pertinent to our subsequent recalculations and investigations.

The theories of Ellis and von Smoluchowski (based upon an old observation of Quincke) have made possible the quantitative measurements of cataphoretic mobility. Ellis assumed that for a closed flat cataphoresis cell of depth x, the observed cataphoretic velocity of a particle was, at any level in the cell (for a system with no turbulence),

$$V_{obs.} = V_p + V_w \tag{2}$$

(V_p = absolute mobility relative to the liquid due to the charge, constant at all levels; V_w = the velocity of the liquid.) The velocity of the liquid, as is well known, may vary from level to level so that if the electroendosmosis be in one direction, the return flow in the mid-regions of the closed cell must be in the opposite direction in closed systems like those considered. $V_{\text{obs.}}$ is, therefore, a function of the liquid streaming. The absolute velocity of a particle is, then, the mean velocity, M, of the particle within the cell,

$$M = \frac{1}{x_1}\int_o^{x_1} V_{\text{obs.}}\, dx \tag{3}$$

Substituting (2) in (3)

$$M = \frac{1}{x_1}\int_o^{x_1} (V_p + V_w)\, dx = V_p + \frac{1}{x_1}\int_o^{x_1} V_w\, dx \tag{4}$$

For a closed cell $\frac{1}{x_1}\int_o^{x_1} V_w\, dx = 0$ and since V_p is a constant for a given field strength,

$$M = \frac{1}{x_1}\int_o^{x_1} V_{\text{obs.}}\, dx = V_p \tag{5}$$

By measuring V at various levels, V_p may be calculated from the analytic expression relating $V_{\text{obs.}}$ to x, or V_p is readily obtained by graphical integration.

Von Smoluchowski simplified the method adopted by Ellis by proposing that

$$V_p = \frac{3}{4}V_{\frac{1}{4}} + \frac{1}{4}V_{\frac{1}{4}} = V_{\frac{1}{2}} = V_{\frac{1}{4}} \tag{6}$$

where the small sub-numerals represent level $\left(\text{level} = \frac{\text{Depth}}{\text{Total Depth}}\right)$ in the cataphoresis cell. From the foregoing it can also be readily shown that

$$V_E = 2(V_{\frac{1}{2}} - V_p) \tag{7}$$

The data of Ellis and of Svedberg and Andersson have amply confirmed von Smoluchowski's theory for the simple types of systems used by

Ellis and by Svedberg and Andersson. Their experiments are in accord with Equation (6) in that

$$V_{.2} = V_{.3} = (V_p) = \frac{1}{x_1}\int_o^{x_1} V_{\text{obs.}}\, dx \tag{8}$$

for flat cells from 50μ to about 1.0 mm. This means that *in cataphoresis cells of this type and these depths the mobility is constant at a given level. And conversely, if flat cells of different depths have mobilities in agreement with Equations (6) and (8), then the absolute mobilities of the particles measured are governed by Equations (6) and (8).* This is of the utmost importance in the recalculation of van der Grinten's data.

TABLE I

Recalculation of van der Grinten's Data

V_E is calculated by means of Equation (4)

Curve No.	$V_{\frac{1}{2}}$ μ/sec.	V_E μ/sec.	$V_{(\frac{1}{2}V_{\frac{1}{2}}+\frac{1}{2}V_{\frac{1}{2}})}$ μ/sec.	V_E μ/sec.	$V_{\text{Graphical integration}}$ μ/sec.	V_E μ/sec.	Mean R
1 (Fig. I)	2.7	6.6	2.8	6.6	2.8	6.4	2.4
2 (Fig. I)	2.7	6.2	2.8	6.0	2.9	5.8	2.15
3 (Fig. I)	2.7	7.2	3.0	6.6	2.9	6.8	2.4
Fig. 4, van der Grinten, p. 228	2.5	7.2		2.5	7.4	6.8	2.8

IV

Recalculation of van der Grinten's Data

It has been mentioned that van der Grinten found $R = 1.5$ approximately. The data submitted by van der Grinten is of the type given in Fig. I which is reproduced from the paper of this author. Curves 1, 2, 3, and 4 in Fig. I demonstrate that when van der Grinten's cataphoresis cells were more than 0.52 mm. thick, the cataphoretic velocity of the particle (as well as the endosmotic velocity) remained practically constant for the same level in cells of different thicknesses. This does not mean, as van der Grinten interpreted it, that V_p, the absolute speed of the particle, is that found in the mid-regions of the cells. It is rather, as Table I demonstrates, a further confirmation

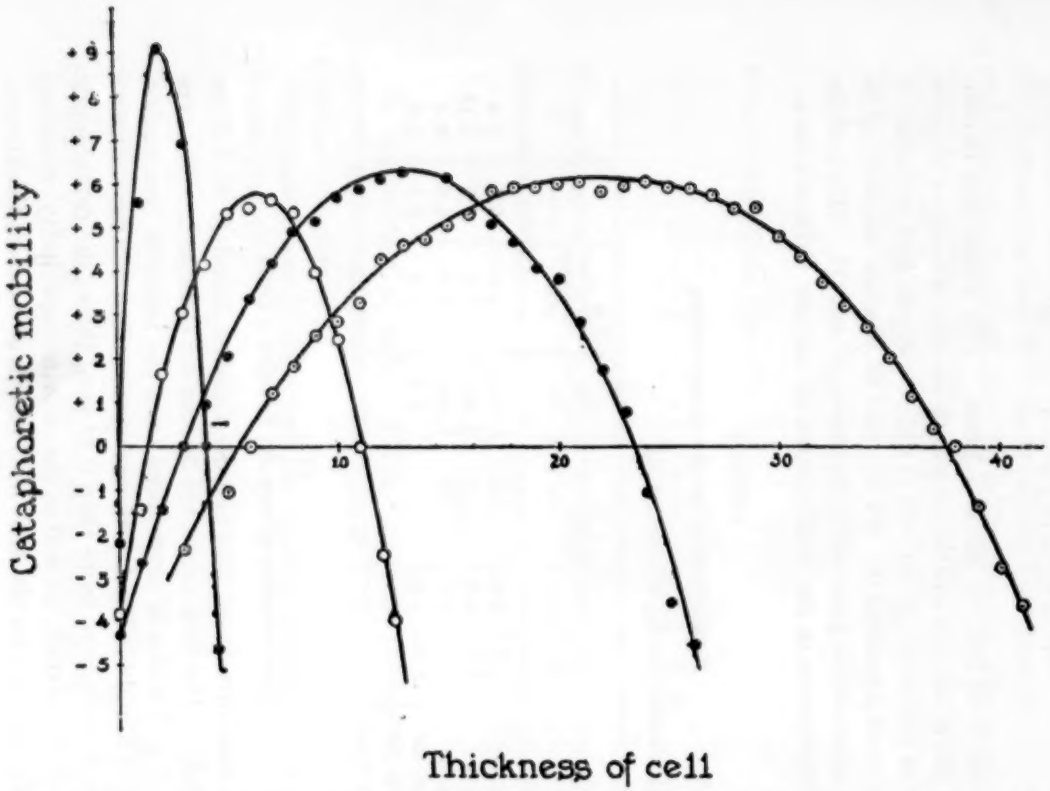

FIG. I. Curves from van der Grinten (6). Electric mobility is plotted against *thickness* of cell for four different cells varying in thickness as indicated. It can be readily calculated from the three curves on the right that the velocity in each case, is, however, practically constant for a given *level* in each cell—confirming, therefore, the theory of von Smoluchowski which may be used to calculate the absolute values of V_p and V_E. Table I and the text indicate the significance of these recalculations.

of the theory of von Smoluchowski. The table gives the values of V_p and V_E calculated from the curves of Fig. I and another curve of van der Grinten's not reproduced here by Equations (6), (7), and (8). It is evident that R is much greater than 1.5 in these systems and varies between 2.15 and 2.8. These data so calculated are in agreement with the author's previous experiments where similar high values of R for glass particle-glass surface systems similar to those of van der Grinten's were obtained. If one considers the data submitted by Lachs and Kronman (12) one can postulate *a priori* that to determine R by means of a flat glass surface and glass particles will be impossible. Lachs and Kronman concluded, after a series of careful streaming potential measurements on glass and quartz surfaces that no true electrokinetic equilibrium was reached. Further, consideration of the well known sensitiveness to stresses of metal surfaces as determined by measurements of thermodynamic potentials makes it not unlikely that localized changes in surface energy due to pulverization of the glass lead to the high value of R. To determine R, a stable system was here sought where R would be independent of the electrolyte content of the medium, and where, with a reasonable degree of certainty the particle surface and the flat surface were the same.

V

The Determination of $\frac{V_E}{V_p}$ *for Flat Surfaces*

It has been shown by Davis (13), Abramson (14), (4), Freundlich and Abramson (4) that surfaces of quartz and glass are practically completely coated with certain proteins when in contact with dilute solutions of these proteins on both sides of the isoelectric point—the particles then acting very much like the native protein in cataphoresis experiments. The most varied substances in addition to quartz and glass coat themselves with gelatin and egg albumin. Thus cystine crystals, menthol, camphor, oil droplets, agar, charcoal, zinc oxide powder and air bubbles behave in this fashion. Briggs (15) has also found that glass capillaries coat themselves with proteins. (His data will be considered in the section on round cataphoresis cells.) By suspending glass, quartz and other particles in a protein solution both particles and flat surface of the cataphoresis cell can, therefore, be

coated with the same substance fulfilling the condition of chemical similarity of glass and particle surface.

TABLE II

Experiments to Determine $\frac{V_E}{V_p}$

G = fused glass cell. C = cemented cell. V_p was taken as $\frac{V_{0.2} + V_{0.8}}{2}$.

Exper. No.	Cell	Nature of system	For a given value of		$R = \frac{V_E}{V_p}$
			V_p μ/sec.	V_E μ/sec.	
1	G	Glass of cell, powdered. pH = 3.6. N/50 Acetate buffer + 0.1 per cent gelatin	11.1	11.8	1.08
2	C	0.004 N HCl + quartz powder + 0.1 per cent gelatin	6.4	6.2	0.97
3	G	0.004 N HCl + quartz powder + 0.1 per cent gelatin	8.7	8.3	0.95
4	G	Benzyl alcohol + 0.2 per cent gelatin	6.0	5.4	0.90
5	G	"	data misplaced		0.97
6	G	Powdered glass in distilled water*	7.3	20.4	3.3
7	G	Quartz in M/150 pH 7.4 phosphate buffer + ½ per cent gelatin	1.23	1.28	1.12
8	C	"	8.61	7.8	0.91
9	C	"	9.6	9.1	1.06
10	C	As above but in dilute acetic acid	10.0	7.8	0.78
11	C	"	10.0	9.6	0.96
12	C	"	10.5	11.4	1.08
13	G	0.1 per cent egg albumin + quartz in M/50 acetic acid	3.2	3.3	1.06
14	G	"	10.3	11.8	1.14
15	G	As above but in phosphate buffer	9.75	11.1	1.14
16	G	½ per cent gelatin + N/200 H_2SO_4	6.15	5.9	0.96
17	G	"	12.0	13.9	1.16

For protein coated surfaces Mn. $R = 1.01 \pm 0.088$ (except No. 10). Probable error, ± 0.02.

* Just one of this type of experiment is indicated here. The results were always similar.

In the experiments to be reported, R was determined in two different flat cataphoresis cells of uniform cross-section. One of the cells was a cemented cell, similar in arrangement to that described by Northrop

(16) and constructed in the fashion previously described. The approximate dimensions of this cell were: length 7.0 cm.; thickness 0.1 cm.; width 1.0 cm. The second cell, of fused glass, was the modification of the Northrup-Kunitz cell described by Abramson (11). The approximate dimensions of this cell[2] were: length 3.5 cm.; thickness 0.08 cm.; width 0.9 cm.

It has been demonstrated for this type of flat cataphoresis cell that "the movements of the water and particle within the cell follow the theory of von Smoluchowski. . . . . When the curve of particle velocity at different levels is parabolic, the curve of velocity as plotted against level is the same near the fused ends of the cell itself as in the middle. The stream lines of the liquid throughout the cell are therefore uniform." The value of R may, therefore, be readily calculated by means of Equations (6), (7), and (8).

Table II gives the values of R for 16 experiments performed with various protein covered particles and the flat glass surfaces of the cataphoresis cells covered with the same proteins. These experiments were performed with two kinds of proteins on both sides of the isoelectric points of the proteins and in the presence of different cations and anions. The field strengths were also varied. The values of R for 15 of these experiments varied between 0.90 and 1.16. The sixteenth value was 0.78. The mean excluding the value 0.78 was equal to 1.01 ± 0.088 with the probable error of the mean equal to ± 0.02. These data point clearly to the conclusion that, under the given conditions, the ratio of cataphoretic to electroendosmotic velocity is very close to 1.00; and that the factor C, in Equation (1) is the same for V_p and V_E.[3]

[2] The diameter of the side tubes connecting cataphoresis cells and stopcocks were large in comparison with the thickness of the cells themselves.

[3] An experimental value of this factor is unknown.

VI

The Determination of $\frac{V_E}{V_p}$ *for a Round Surface*[4]

By proceeding as in Part I, $\frac{V_E}{V_p}$ was determined in a round microcataphoresis cell by coating particle and glass surface with a protein, here gelatin. The curve drawn through the points in Fig. II is typical of the data of four similar experiments. The absolute mobility of the protein covered particle, V_p, is near the level 0.15. The curve passes through the origin. This is only possible when

$$V_E = V_p \text{ or } \frac{V_E}{V_p} = 1.00$$

The dotted line in Fig. II follows the course of the curve calculated on the assumption that $\frac{V_E}{V_p} = 1.5$. That this ratio does not obtain under these conditions is obvious, confirming, therefore, the data for flat cells.

The data presented in the previous sections fit in with the interpretation of the evidence submitted previously (3) that

$$V_p = \frac{C}{\pi} \frac{DX\zeta}{\eta}$$

where C is a constant independent of the size and shape of the particle. The experimental investigations submitted here and previously have demonstrated that within experimental error

$$V_p = V_E$$

where the radius of the particle varies from about 0.5μ to 100μ, possibly as low as 0.010μ for certain proteins.

[4] Briggs has found that streaming potentials obtained with protein coated quartz surfaces agree with mobilities obtained by the method of cataphoresis. This is evidence that $\frac{V_E}{V_p}$ would be 1.00 for round surfaces, but since different electrolyte concentrations were used this conclusion cannot be made before conditions are made more nearly alike.

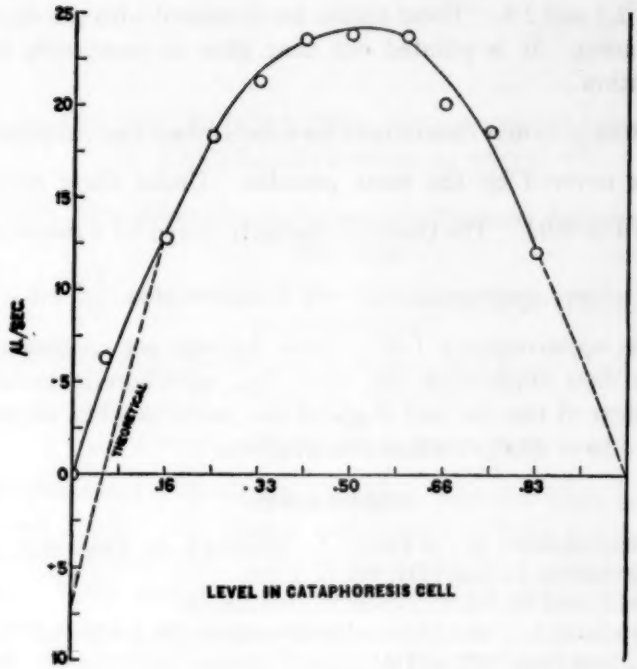

FIG. II. The smooth curve extrapolated to $x = 1.0$ has been drawn through points of cataphoretic mobility of protein covered oil droplets. The curve passes through the origin. Since the origin ($x = 0$ or 1.0) is the wall of the cell, it follows that $V_E = V_p$ or $\frac{V_E}{V_p} = 1.00$, approximately. The arrow points to the value of V_p obtained as described in the text. The dotted line is the course of the curve predicted by the theory of Debye and Hückel for a *flat* surface. This curve has been obtained with a round cell.

SUMMARY

Two theories which predict different values for the ratio of V_E, the electroendosmotic velocity of a liquid past a surface, to V_p, the electric mobility of a particle of the same surface through the same liquid are discussed. The theory demanding that $\frac{V_E}{V_p} = 1.5$ was supported by certain data of van der Grinten for a glass surface. Recalculation of van der Grinten's data reveals that the ratio varies

between 2.1 and 2.8. These results are in accord with previous data of Abramson. It is pointed out that glass is unsuitable for the investigation.

The ratio $\frac{V_E}{V_p}$ is here determined for a flat surface and particles when both are covered by the same proteins. Under these conditions $\frac{V_E}{V_p} = 1.01 \pm 0.02$. The theory is similarly tested for a round surface using a micro-cataphoresis cell. It is shown that $\frac{V_E}{V_p}$ for a round surface is approximately 1.00. These findings are confirmatory of previous data supporting the view that cataphoretic mobility is independent of the size and shape of the particles when all particles compared have similar surface constitutions.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

IV. The Isoelectric Points of Certain Sensitized Antigens

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The behavior of antibodies in an electric field was early studied by Field and Teague[1] and Teague and Buxton.[2] They concluded that the antibodies migrated to the cathode and were therefore electropositive. Using similar apparatus Maver and Falk[3] have since reproduced these results and shown them to have been due to failure to take account of electroendosmotic streaming through the agar gels used. Eliminating this error, Maver and Falk have found that diphtheria antitoxin migrated to the anode at pH 6.0 or more alkaline reactions, and to the cathode at pH 4.6 or more acid reactions. Landsteiner and Pauli[4] showed that hemagglutinins behaved in an electric field as ampholytes.

Michaelis and Davidsohn,[5] using macrocataphoresis, found the isoelectric point of typhoid agglutinin to be between hydrogen ion concentrations of 1.0 and 5.1 $\times$ 10^{-6}. This range agreed with the isoelectric range for serum globulins as earlier determined by Rona and Michaelis;[6] their mean value of 3.6 $\times$ 10^{-6} (pH = 5.4) for serum globulin is usually cited as the isoelectric point of globulin. Von Szent-Györgyi[7] confirmed the value for the isoelectric point of typhoid agglutinin found by Michaelis and Davidsohn.

The isoelectric point of typhoid agglutinin was reinvestigated by Ottenberg and Stenbuck,[8] using both agglutinins in immune serum and relatively pure agglutinins dissociated from the sensitized bacteria. They obtained values for the isoelectric point between pH 4.4 and

pH 4.6. However Ottenberg and Stenbuck used citrate buffers. Michaelis and Rona[9] showed that the isoelectric point of a protein could be shifted toward the acid side by almost a full pH unit by polyvalent anions such as citrate. The effect was later given theoretical treatment by Michaelis.[10] The shift of the isoelectric point toward the acid side by citrate has been confirmed by one of us, using animal membranes,[11] and in this present study using sensitized bacteria and serum globulin. The discrepancy between the isoelectric point of typhoid agglutinin as found by Ottenberg and Stenbuck and by earlier investigators is thus certainly due in whole or in part to the use by the former of citrate buffers.

Study of the cataphoretic behavior of antibody when combined with its corresponding antigen was undertaken by Coulter.[12] He reported the isoelectric point of sheep red blood cells to be at about pH 4.6 and their optimum for acid flocculation to be at about pH 4.75. After heavy sensitization with immune serum the agglutination optimum was shifted to about pH 5.3. Coulter reported no corresponding shift of the isoelectric point, however, with serum sensitization. However, the description of his technique suggests that the erythrocytes used in the cataphoresis experiments were only lightly sensitized. It will be apparent from the graphs in this paper that submaximal sensitization may readily lead to attributing too alkaline an isoelectric point to the sensitizing substance.*

Northrop and De Kruif,[13] in a study which gave the first adequate experimental basis for analysis of the mechanism of agglutination, showed the isoelectric point of typhoid bacilli to be progressively shifted toward the alkaline side by increasing concentrations of sensitizing serum. Their curves (Fig. 3) show sensitization with no greater concentration of serum than 1:150, however, and undoubtedly the isoelectric point of 4.7 attributed to the sensitized bacteria is on the acid side of that which would have been reached with maximal sensitization.

* Dr. Coulter has informed one of us that in later, unpublished work he found the cataphoretic isoelectric point of more strongly sensitized sheep erythrocytes to have been shifted to about pH 5.3. In our own work sensitization with our best immune serum shifted the isoelectric point of sheep erythrocytes to pH 5.7; this will be reported in detail in a later paper.

Shibley[14] found that colon bacilli sensitized with homologous agglutinating serum in 1:20 dilution were isoelectric at about pH 5.3. The cataphoretic velocity of colon bacilli and pneumococci so sensitized varied with respect to pH and molar concentration of the suspending buffers almost precisely like denatured serum globulin.

EXPERIMENTAL

Bacteria Used.—An avian tubercle bacillus, *Mycobacterium avium* (Arloing strain), and the turtle bacillus, *Mycobacterium chelonei*. These were grown on glycerol-agar slants and suspended in 0.85 per cent NaCl solution. Both microorganisms were acid-fast.

Sensitization.—The bacteria were sensitized with homologous rabbit immune serum or plasma. Plasma was obtained by drawing blood into test tubes containing a few crystals of K-oxalate; the plasma was centrifugalized and the supernatant used. One volume of bacterial suspension was added to one volume of each serum or plasma dilution; the abscissae on the graphs are the serum or plasma dilutions after mixing. The mixtures were kept in the ice-box over night. They were centrifugalized, the supernatant fluid decanted, the sediment resuspended in excess of 0.85 per cent NaCl solution, again centrifugalized, decanted and resuspended in fresh NaCl solution. One or two drops of these suspensions of sensitized, washed bacteria were added to each 3 cc. of buffer solution.

Globulins.—Euglobulin was obtained from immune sera by simple dilution with distilled water and chilling, or preferably by dilution with fifteen volumes of distilled water and acidification drop by drop with N/10 hydrochloric or acetic acid. The precipitate formed by slight acidification was thrown down by centrifugalization, and the supernatant decanted; the sediment was resuspended in excess of distilled water; again thrown down, decanted, and resuspended in distilled water. A few drops of the latter suspension were added to dilute buffer solutions for study by cataphoresis. Euglobulin and pseudoglobulin were also prepared by $(NH_4)_2SO_4$ fractionation as described elsewhere.[15] The euglobulin and pseudoglobulin solutions prepared by $(NH_4)_2SO_4$ fractionation were evaporated to dryness in Petrie dishes; the protein was subsequently scraped from the bottom of the dish and suspended in distilled water. A few drops of these suspensions were added to dilute buffers for cataphoresis.

Buffers.—Walpole's acetate, Sørensen's phosphate and McIlvaine's citrate-phosphate buffers were made up according to the tables given by Clark.[16] They were either used in the original strength, *i.e.*, for Walpole's M/5, for Sørensen's M/15, for McIlvaine's M/5 to M/10, or diluted with nine volumes of distilled water added to each volume of buffer. In the extreme acid range phthalate-HCl or KCl-HCl mixtures or dilute HCl were used. The pH of the buffers or buffer dilutions used were checked colorimetrically.

Cataphoresis.—The Northrop-Kunitz microcataphoresis cell as modified by

Kunitz[17] was used with a dark field condenser and a Bausch and Lomb, 8 mm., 0.50 n.a., 21 × objective. Three readings at the lower and three at the upper "stationary level,"[18] *i.e.*, at 0.21 and 0.79 of the inside depth of the cell, were made for each suspension. Radio B-batteries were used; the applied potential was 135 volts, giving a gradient through the cell of about 7 volts per centimeter.

Estimations of Isoelectric Point.—Buffer series were made up so that the successive members differed by 0.4 pH in the case of Walpole's and Sørensen's, by 0.6 pH with McIlvaine's. Cataphoresis determinations were made upon a drop or two of bacterial or globulin suspension in 3 cc. of buffer, until two successive buffers were found in one of which the particles migrated toward the anode, in the other toward the cathode. The isoelectric point was then estimated by interpolation. It will appear later that the important comparisons to be made in this study were between the isoelectric points in acetate or phosphate buffers of strongly sensitized bacteria and of globulin precipitated chemically from the sensitizing serum. These comparisons, we believe, were usually valid to within 0.1 pH. When comparing results of different experiments absolute errors greater than 0.1 pH were no doubt sometimes introduced by inexactitude in determining the pH of the buffers, by changes in the cataphoresis cell due to deposit on its walls of substances from the test suspensions, and to errors in focussing.

The type of result obtained is shown in Fig. 1. The unsensitized bacillus is negatively charged except at very acid reactions. Sensitization with increasing concentrations of homologous immune serum shifts the isoelectric point* progressively toward the alkaline side until it reaches and passes beyond the isoelectric point of globulin precipitated by acidification or salted out of the same immune serum. In the experiments graphed the concentration of immune serum required to bring the microorganisms to the isoelectric point of serum globulin, represented by the horizontal lines at pH 5.2, was between 1 part serum in 64 and 1 part serum in 16 of diluent. At concentrations of immune serum of 1 part in 16 or higher the isoelectric point of the sensitized bacteria was *definitely higher than that of the globulin.*

The progressive change in cataphoretic properties of the bacteria with sensitization may also be followed by determining the cataphoretic velocity at a constant pH. In buffers of pH 5.2 the anodal migration velocity of the bacteria is progressively reduced by sensitization with increasing strengths of immune serum, and in the highest

* "Isoelectric point" is in this paper used in the original empirical sense of Hardy as the hydrogen ion concentration at which no migration in an electric field occurs.

serum concentrations migration becomes cathodal. Quantitatively parallel to these changes in cataphoretic properties other changes regularly occur with sensitization. The bacteria cohere more strongly (positive resuspension and interface reactions), they show increasing resistance to passage through a phase boundary from aqueous to oil phase (positive interface reaction), they become agglutinated, and in

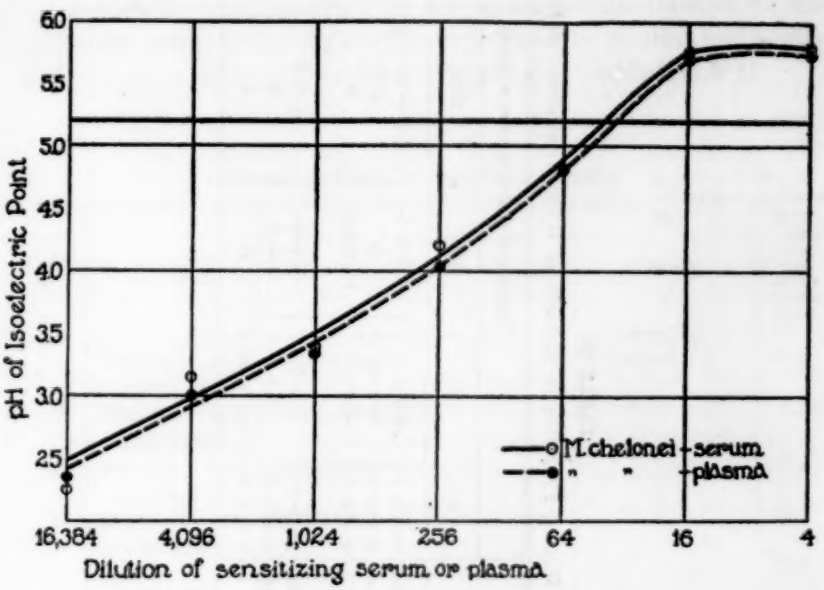

FIG. 1. Progressive change of the isoelectric point of an acid-fast bacterium with sensitization. The isoelectric point of the unsensitized bacterium is below pH 2.5. After exposure to homologous immune serum or plasma in progressively increasing concentrations the isoelectric point shifts to pH 5.7. The horizontal line at pH 5.2 is the mean value for seven determinations of the isoelectric point of serum euglobulin.

the presence of leucocytes, they are phagocyted. Abundant evidence has been submitted by us[18,19] to show that these changes are all a result of the deposit on the bacterial surface of a substance or substances present in the globulin fractions of immune serum. The combination of immune protein with antigen is mediated by specific chemical affinities; the results, *i.e.*, agglutination, the altered surface properties

TABLE I

Agglutination, Changes in Cohesion and Wetting Properties in Experiment Whose Isoelectric Points are Plotted in Fig. I

	Reactions	Dilutions of sensitizing serum or plasma						
		1:4	1:16	1:64	1:256	1:1,024	1:4,096	NaCl control
M. chelonei sensitized with anti-chelonei Serum 2637 and washed	Agglutination	++++	++++	++++	++++ to +++	+++	0	0
	Resuspension	++++	+++	+	++	tr.	tr.	0
	Interface	++++	+++	+ (weak)	0			0
M. chelonei sensitized with anti-chelonei Plasma 2637 and washed	Agglutination	+++	+++	+++	+++	++	0	0
	Resuspension	++++	++	+++	+	tr.	0	0
	Interface	++++	+++ to ++	0	0			0

and phagocytosis, are all dependent upon the properties of the substances from the serum combined with and deposited upon the antigen surface.

In Table I are given the agglutination results and changes in cohesion and wetting properties in the experiment whose isoelectric points are plotted in Fig. I.

The antibody-protein against acid-fast bacteria is found chiefly in the euglobulin, to a less extent in the pseudoglobulin fraction.[18] The isoelectric points of the euglobulin precipitated by dilution and acidification from immune sera used in this study are given in Table II.

TABLE II

Isoelectric Points of Euglobulin Samples

Experiment No.	Method of preparation	Serum	Globulin	Isoelectric point with acetate or phosphate
1	Dilution and acidification	AntiArloing 35	Unheated	5.2
2	Dilution and acidification	Antichelonei 2637	"	5.25
3	Dilution and acidification	Antichelonei 39	"	5.3
4	Dilution and acidification	AntiArloing 35	"	5.2
5	Dilution and acidification	Antichelonei 2637, heated 56° for 30 min.	"	5.0
6	Dilution and acidification	Antichelonei 3293, heated 56° for 30 min.	"	5.1
7	Dilution and acidification	AntiArloing 35, heated 56° for 30 min.	"	5.4
8	Dilution and chilling	AntiArloing 2226	Heated 97°–98° for 10 min.	5.15
9	Dilution and chilling	Antichelonei 2213 and 2228	Heated 100° for 15 min.	5.15
10	Dilution and acidification	Antichelonei 2637, heated 56° for 30 min.	Heated 100° for 15 min.	4.55
11	Dilution and acidification	Normal Serum 3373	Unheated	5.05
11	Dilution and acidification	" " "	Heated 87°–92° for 15 min.	5.1
12	Dilution and acidification	Normal Serum 3369, heated 56° for 30 min.	Heated 87° for 20 min.	5.05

The mean of the first seven values in Table II is plotted in Figs. 1 and 2 as the horizontal line at pH 5.2. These were all euglobulin samples freshly precipitated from immune sera and subjected to no heating or drying, except in three instances the heating involved in inactivation of the immune serum. Eleven additional euglobulin samples have been precipitated by dilution and acidification or salted out[25] of anti-protein precipitin rabbit sera. The range of isoelectric points for these eleven samples was pH 4.9 to pH 5.2, with a mean value of 5.1. Eleven isoelectric point estimations have also been made

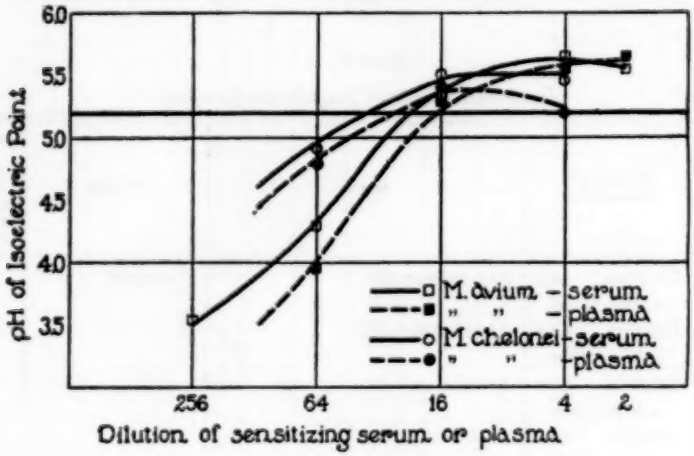

FIG. 2. Progressive change of the isoelectric points of acid-fast bacteria with sensitization. Exposure to homologous immune serum or plasma progressively shifts the isoelectric points until they pass to the alkaline side of that of serum globulin, indicated by the horizontal line at pH 5.2.

upon pseudoglobulin fractions salted out of rabbit sera, dried and kept for varying lengths of time. The isoelectric points found for these pseudoglobulins ranged from pH 4.4 to pH 5.3 with a mean of 4.9; however drying and aging may have shifted the isoelectric point of some of these pseudoglobulin samples somewhat toward the acid side* as heating seems to have done for one or more of the euglobulin samples in Table II.

* We are informed by Dr. L. D. Felton that drying regularly shifts the point of minimum solubility of horse globulin toward the acid side.

The isoelectric points of sensitized bacteria in two other experiments are graphed in Fig. 2. The features to be emphasized are again (1) that the isoelectric point of the sensitized antigen shifts toward the alkaline side with increasing concentration of sensitizing serum or plasma, and (2) that in the highest concentrations of serum the isoelectric points reached are slightly but distinctly above the values for

TABLE III

Comparison of Isoelectric Points with Buffers Containing Monovalent and Trivalent Anions

Experiment No.	Test object	Dilution of sensitizing serum	Isoelectric point with acetate or phosphate buffer pH	Isoelectric point with citrate buffer pH
5	*M. chelonei*	1:2	5.7	5.05
	" "	1:4	5.7	—
	" "	1:16	5.1	—
	" "	1:∞	2.8 or lower	
	Euglobulin from sensitizing serum		5.0	4.6
6	*M. avium*	1:2	5.05	4.45
	" "	1:4	5.0	—
	" "	1:16	4.8	—
	" "	1:64	3.8	—
	" "	1:∞	not reversed in 0.1 N HCl	
	Euglobulin from another antiserum		5.1	4.45
12	*M. chelonei*	1:2	5.55	5.0
	" "	1:4	5.35	—
	" "	1:16	4.8	—
	Euglobulin from normal serum		5.05	4.4

the isoelectric points of the precipitated or salted-out euglobulin and pseudoglobulin.

It seemed that the explanation of the isoelectric point of strongly sensitized bacteria being on the alkaline side of serum globulin might conceivably lie in deposition on the bacteria from the lower serum dilutions of some residual fibrinogen. It was to test this possibility that bacteria were sensitized with plasma as well as with serum.

Plasma, however, gave no higher, indeed not as high values as serum, thus lending no support to this hypothesis.

Isoelectric points found for sensitized bacteria and for globulin in three other experiments are given in Table III. In two of these values for the strongly sensitized bacteria are found to be well on the alkaline side of the globulin; in Experiment 6 the isoelectric points of the most strongly sensitized bacteria and of the globulin coincided.

The shift of the isoelectric points toward the acid side by the use of a buffer containing polyvalent anions is shown in Table III. The buffers used in this experiment were about one-twentieth molar with respect to citrate; the isoelectric points both of the sensitized bacteria and of the euglobulin are seen to be about one-half pH unit more acid than in the corresponding acetate or phosphate mixtures.

DISCUSSION

Unsensitized acid-fast bacteria have very low isoelectric points and are wetted by oils in a manner indicating a high lipin content for their surfaces. As these bacteria interact with increasing concentrations of immune sera their surface properties progressively change. The strongly sensitized bacterial surfaces have wetting properties like those of protein,[20] are cohesive,* and are isoelectric at reactions slightly more alkaline than those of precipitated serum globulin; concomitantly with the changes in surface properties the bacteria are agglutinated* and prepared for phagocytosis.

For comparison consider the effect of sensitizing an essentially different type of antigen. Loeb[22] found that collodion particles could be coated with protein, and then behaved in an electric field like particles of the pure protein. This device has been adapted to serological uses by F. S. Jones,[23] and independently by Freund.[24] We have deposited crystalline egg albumin, edestin and human serum proteins on collodion particles. These particles were then agglutinated and prepared for phagocytosis by the sera of rabbits immunized with the corresponding proteins.[25] As such protein treated collodion

* In the formulation of Northrop,[21] with which this work is essentially in harmony, the critical potential of the antigen is increased by deposit of a surface film of agglutinin.

particles were sensitized with increasing concentrations of homologous immune serum their isoelectric points progressively shifted to the alkaline side until the same values were attained as in the case of sensitized acid-fast bacteria.

In general, as far as present evidence goes, though this is still incomplete, the surface properties of diverse antigens have been found to converge with progressive sensitization toward that condition described for strongly sensitized acid-fast bacteria. There is no doubt, at least in our minds, that the properties of the maximally sensitized antigens are or approximate those of the sensitizing substance or substances of the immune serum *after deposition upon the antigen surface.** In brief the sensitizing protein forms a surface deposit on the antigen with which it specifically combines. The combination depends upon the specific chemical affinities of antigen with antibody. The surface properties, agglutination and phagocytosis of the maximally sensitized antigen depend chiefly upon the serum substances combined with and deposited on the antigen surface.

Felton[26] has succeeded in bringing the antibodies of anti-pneumococcus horse sera to a state of relative purity. He believes his best preparations to consist of about equal parts of active antibody-protein and inert serum globulin. The antibody-protein itself seems to be essentially a modified globulin or something at least in considerable part made up of globulin. Felton has reported the point of minimum solubility of his antibody-protein to be pH 6.6 to 6.8.

Is the isoelectric point of pH 5.6 to 5.8 we have found for antigens strongly sensitized with rabbit sera to be regarded as the isoelectric point of rabbit antibody-protein? Or is this value due to a mixed deposit of inert serum globulin with an antibody protein of more alkaline isoelectric point analogous to Felton's? Why should the antibody protein, which is found in the globulin fractions of serum, have a higher isoelectric point when combined with antigen than the globulin precipitated chemically from the same serum? Is there an active substance of higher isoelectric point mixed with the inert globulin or does the antibody-protein change its isoelectric point in

* There is evidence that the antibody-protein undergoes denaturization on combination with the antigen surface; see references 14 and 20, also a paper by H. Eagle which appeared when this paper was in press, (*J. Immunol.*, 1930, **18**, 169).

combining with antigen because of altered orientation or the combination of certain radicals? Answers to these questions must wait upon further investigations.

CONCLUSIONS

Sensitization with increasing concentrations of homologous immune serum shifts the isoelectric point of the antigens studied progressively to the alkaline side. Antigens maximally sensitized with rabbit sera have shown isoelectric points of pH 5.6 to 5.8. The globulins precipitated or salted out of the same immune sera have been isoelectric at pH 5.1 to 5.2.

The combination of antigen with antibody depends of course upon specific affinities; the surface properties of the sensitized antigen, agglutination and phagocytosis depend primarily upon the properties of the sensitizing serum substances combined with and deposited on the antigen surface.

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RESPIRATION OF MAMMALIAN ERYTHROCYTES

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1. Respiration of Erythrocytes Induced by a Respiratory Supplement Prepared from Various Tissues

Lavoisier's fundamental interpretation of respiration as oxidation of organic compounds by free oxygen is valid even at the present time without restriction. His opinion was that this oxidation takes place in the blood. This hypothesis had to be given up, the respiration really taking place in the tissues. Red corpuscles, at least the non-nucleated corpuscles of mammals, do not participate in the reduction of oxygen to any appreciable extent.

Recently, however, Harrop and Barron (1, 2) showed that non-nucleated erythrocytes do respire in the presence of methylene blue and some other related dye stuffs, which can be designated, therefore, as catalysts for respiration. All of these artificial catalysts are alien to the animal body, and no component of any tissue has ever been shown to exhibit the same effect as methylene blue on erythrocytes. Never has a respiration of non-nucleated erythrocytes in any way comparable in extent to that of other tissues been observed prior to the experiments of Harrop and Barron.

It will be shown in this paper that a substance can be extracted by water from various organs which induces non-nucleated erythrocytes to respire just as methylene blue does. This substance will be shown not to be identical with Warburg's (3) respiration enzyme because it is not sensitive to carbon monoxide.

Methylene blue was used by Thunberg (4) as an oxidant instead of oxygen, for something that may be reckoned as respiration only when the meaning of this concept is considerably widened. In his experiments, methylene blue is a hydrogen acceptor, instead of oxygen,

which is absent throughout the experiment. Different from these experiments is the use of methylene blue in the presence of oxygen as catalyst for the consumption of oxygen by cells. Meyerhof (5) was the first to show that methylene blue sometimes acts as a catalyst for respiration in air. He observed this property of methylene blue, however, only in cell extracts the respiratory faculty of which had been artificially damaged, such as in heated extract of yeast previously treated with acetone. The respiration of the yeast cells themselves could not be improved by methylene blue under normal conditions. Not even the oxygen consumption of the extract of acetone yeast was altered by methylene blue unless the spontaneous oxygen consumption of such an extract was damaged by heating. Only a damaged respiration could be improved by methylene blue, this dye acting as a substitute for the destroyed respiration enzyme.

Later on it was shown by Harrop and Barron (2a), that certain cells which by nature exhibit a very low respiration, or none at all under natural conditions, can be stimulated to an intense respiration by methylene blue. This has been shown for erythrocytes and for unfertilized echinoderm eggs. The effect of methylene blue, according to Barron, is shown also for other dyes reversibly oxidizable and reducible. The magnitude of this effect appeared to depend on the potential range of the dye, methylene blue showing the optimum effect, whereas dyes with a more positive or a more negative range of potential have smaller effects or even no effect at all. As for the interpretation of this observation, we may refer to Barron's paper.

The reaction of a physiologist's mind to this observation may be, and has been, different. One point of view is that this effect of methylene blue is an accidental property of the dye, that no substance analogous to the dye exists in the living organisms, as seemingly shown by the fact that erythrocytes really do not respire. Another point of view is the hypothesis that methylene blue might be a model for some unknown substance in the organisms, and that this substance may play a part in respiration, which is not obvious as yet. This hypothesis is supported by the observation presented in this paper, showing that extracts of various organs induce erythrocytes to respire just as methylene blue does. The best effect was obtained with liver extract of various animals, and rat's liver seems to be the most suitable tis-

sue for the extraction of the active substance, to which we will refer in what follows under the term *respiratory supplement.* Then follows kidney, spleen, testicle, lymph-node, whereas brain, muscle, and blood serum showed no effect or at most a very slight effect. Ox organs showed similar effects, the action often being somewhat less intense, but the present method is not yet adapted for a quantitative extraction of the supplement or for the estimation of its original concentration in the living organ.

The respiration of blood corpuscles induced by methylene blue or by organ extract is not influenced at all by the presence of carbon monoxide. Rabbit's erythrocytes, in the presence of methylene blue or rat's liver extract, respire at the same rate in air or in a mixture of pure carbon monoxide containing a small amount of oxygen, varied from 1 to 2 per cent in different experiments. Such a gas mixture inhibited the rate of respiration of yeast or of nucleated erythrocytes of fowl by approximately 60 to 80 per cent depending on the conditions, in accordance with Warburg's observations. This difference in the behavior toward CO proves that the supplement is not identical with Warburg's respiration enzyme.

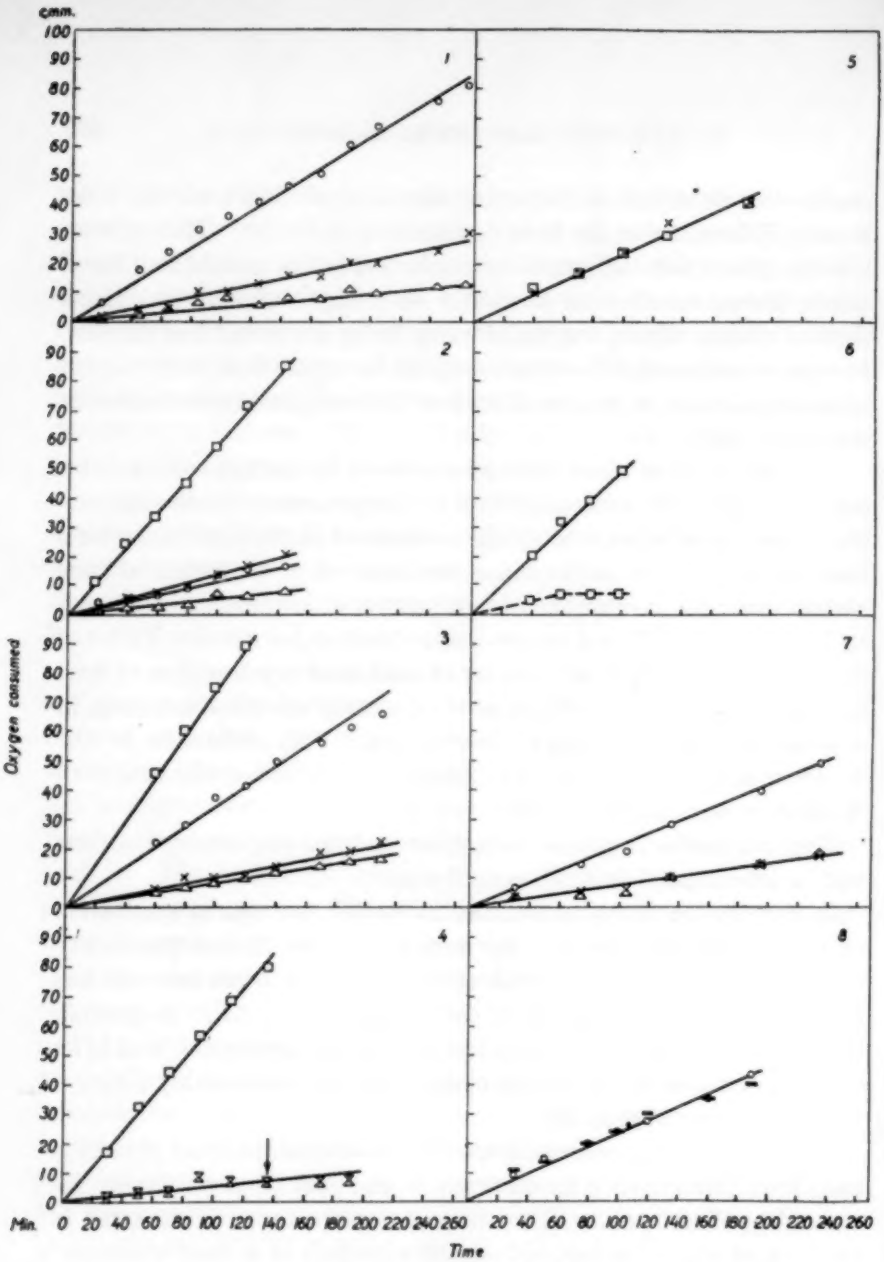
The end products of this respiration and the respiratory quotient will be investigated in a subsequent paper.

In varying the temperature from 23° to 37°, the rate of respiration of blood corpuscles induced by methylene blue showed practically no difference, whereas the respiration induced by organ extracts was two to three times as fast at 37° as compared with 23°. In general, the organ extracts, at 23°C., are less active than methylene blue in its optimum concentration (0.0006 molar), but are considerably stronger than methylene blue at 37°.

As a preliminary statement it may be added that the effect of methylene blue, though very strong even in the dark, is considerably increased by illumination. The effect of organ extracts is the same in the dark or when illuminated by a 150 watt bulb at a short distance.

Methylene blue had no influence upon that slight oxygen consumption exhibited by organ extracts alone in the concentrations used.

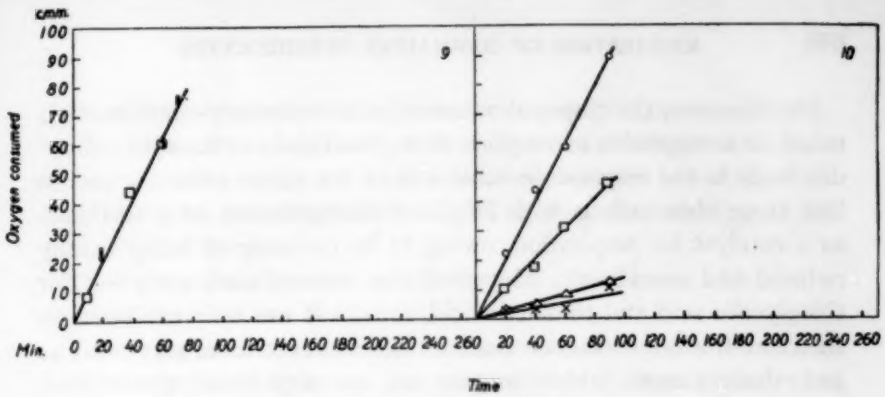
The influence of narcotics has been studied only for phenyl urethane as yet. This showed no influence at all upon the effect of methylene blue or of the supplement.



Figs. 1 to 8 at 23°C.

Common designations for all curves:

- ○ Experiments with blood, sugar and organ extract.
- × Control with blood and sugar.
- △ Control with organ extract and sugar, without blood corpuscles.
- □ Experiment with blood, sugar and methylene blue.
- ⧖ Control with blood plus methylene blue, without sugar.
- - - - Experiments with hemolyzed blood (Fig. 6).
- = Experiments with blood, sugar, organ extract in carbon monoxide containing 1.4 per cent oxygen (Fig. 8).



Figs. 9 and 10 at 37°C.

Common designations for all curves:—*continued.*

‖ Experiments with blood, sugar, methylene blue in carbon monoxide with 1.4 per cent oxygen.

FIG. 1. Effect of rat liver extract at 23°C. The respiration in presence of rat liver extract is far greater than the sum of the respirations in all controls.

FIG. 2. Effect of methylene blue (□) compared with that of rat muscle extract (○), at 23°C., in the presence of sugar. Muscle extracts show no effect in this case (a very slight effect in other cases, especially at 37°).

FIG. 3. Comparison of methylene blue (□) and rat kidney extract (○) at 23°C. At this temperature organ extracts as a rule act less intensely than methylene blue.

FIG. 4. Shows that methylene blue increases the respiration only in presence of glucose (□) whereas it has no influence in absence of sugar (⊠). When sugar is added after the methylene blue has been in contact with the blood corpuscles for 2 hours, subsequent addition of sugar induces no respiration. The arrow shows the time of addition of glucose.

FIG. 5. For chicken erythrocytes respiration is not influenced by the addition of methylene blue even in presence of sugar. Temperature 23°C. The spontaneous respiration of these nucleated erythrocytes in sugar is rather high alone even at 23°C., and cannot be increased by methylene blue.

FIG. 6. The effect of methylene blue is present only for intact erythrocytes but not after hemolysis.

FIG. 7. Effect of rat spleen on the respiration of rabbit erythrocytes in presence of sugar. 23°C. The effect is somewhat smaller than with liver.

FIG. 8. The respiration of rabbit erythrocytes induced by rat liver extract is equal in air (○) or in carbon monoxide containing 1.4 per cent oxygen (=).

FIG. 9. The respiration of rabbit erythrocytes in the presence of glucose induced by methylene blue, is equal in air (□) or in carbon monoxide containing 1.4 per cent oxygen.

FIG. 10. Effect of rat liver extract (○) or of methylene blue (□) on the respiration of rabbit erythrocytes with glucose at 37°. At this temperature the effect of organ extract is greater than that of methylene blue.

On discussing the chemical nature of this respiratory supplement it might be a suggestive assumption that glutathione or another sulfhydril body is the responsible substance of the tissue extracts, and to link these observations with Hopkins' interpretation of glutathione as a catalyst for respiration, owing to its property of being readily reduced and reoxidized. Meyerhof also showed such an effect for thioglycolic acid and thiolactic acid, though it was only exhibited on structure-free cell extracts. Such an idea was even more suggestive as just those tissues which contain no, or very little, glutathione, namely, blood serum and muscle tissue, show no, or a very small, effect on the respiration of blood corpuscles. Our experiments, however, do not as yet support this hypothesis. Reduced glutathione, or cysteine, added to blood corpuscles in Ringer solution plus glucose, consumed just as much oxygen as was required for the oxidation to the disulfide. When added in such small amounts that this oxygen consumption could be practically neglected, they had no effect whatsoever. When glutathione in the oxidized state or cysteine was added, no effect could be seen at all, with or without addition of an iron salt.

A second possibility was that the respiratory supplement might be identical with Harden and Young's (7) coenzyme. Meyerhof showed that the coenzyme enhances not only fermentation but also oxidation. However, this idea has to be abandoned too, because muscle contains by far the greatest amount of the coenzyme among all tissues, according to Meyerhof, whereas our effect is exhibited by muscle extract almost to the least degree among all tissue extracts. The fact that muscle extract contains so little supplement is also contradictory to the suggestion that Keilin's cytochrome be identical with the supplement.

On searching for another explanation, one may think of the fact that it is just cells without a nucleus (erythrocytes) or cells with a nucleus in an obviously resting state (unfertilized eggs) which can be induced to respire by methylene blue. Perhaps the respiratory supplements of organ extracts, analogous to methylene blue, is some substance derived from the cell nucleus. In fact, tissues with no nucleus (serum) or with very few nuclei per unit of mass (muscle, brain), have almost no effect. This idea is, at the present time, a mere suggestion.

The respiration of erythrocytes as described before, either under the

influence of methylene blue or of the supplement, is shown only by intact erythrocytes. Erythrocytes hemolyzed by distilled water, or by repeated freezing, show no respiration, or when the hemolysis is incomplete there is a diminution of the respiration according to the extent of hemolysis.

The respiration of chicken erythrocytes is not influenced by methylene blue.

There is in the literature on respiration one observation which may have a connection with ours. Battelli and Stern (8) showed that what they call the accessory respiration of a tissue or a tissue extract (such as liver or kidney) is noticeably, though not strongly, increased by addition of washed blood corpuscles. This observation, however, is made under rather different experimental conditions and differs from ours by the fact that the effect described by Battelli and Stern is the same whether the blood corpuscles are added in the intact or in the hemolyzed state, whereas our effect is strictly confined to the intact state of the erythrocytes.

2. The Influence of the Medium upon the Action of the Respiratory Supplement

The medium in which most of the experiments were performed is Ringer solution with 0.18 per cent of glucose. In sugar-free Ringer solution methylene blue shows no effect, at least when the erythrocytes are used in not too fresh a condition, where the natural sugar content of the blood corpuscles may be supposed to be not yet consumed. But even in very fresh condition, when perhaps not all of the natural sugar content of the blood corpuscles may have been destroyed, the respiration of blood corpuscles under the influence of methylene blue is very much smaller in sugar-free Ringer solution than it is in a medium containing sugar. When sugar-free blood corpuscles suspended in Ringer solution containing glucose are mixed with methylene blue, a strong oxygen consumption starts immediately and goes on at a constant rate over several hours; indeed over a period of time such an experiment of this sort can reasonably be extended, 5 to 6 hours at 23°, or 2 to 3 hours at 37°. Methylene blue does not harm the blood corpuscles within this period. When, however, methylene blue is added to a suspension of blood corpuscles in a sugar-free

medium, the dye seems to exhibit a damaging influence to the blood corpuscles. For not only is the respiration missing under this condition, but also the respiration is not established on adding glucose after an interval of 1 to 2 hours. One may think of the following interpretation: If there is no sugar the oxidation catalysed by methylene blue is directed toward some other oxidizable substance in the cell which is important for its physiological structure. This substance is protected from oxidation in the presence of sugar.

Experiments of the same kind but with organ extracts instead of methylene blue did not lead to any interpretable result because such extracts cannot be obtained free from sugar or glycogen.

The effect of methylene blue can be shown to the same extent within the limits of error by substituting fructose or saccharose for glucose. In contrast, there was no respiration with mannite. Ringer solution with mannite behaves like sugar-free Ringer solution.

On attempting to replace Ringer solution in the methylene blue experiments by various isotonic salt solutions, the following results were obtained. The oxygen consumption in 0.9 per cent NaCl solutions was somewhat smaller than in Ringer solution. Omitting only $CaCl_2$ from the Ringer solution the respiration was a little smaller too, when compared with the control with complete Ringer solution. By substituting for the very small amount of $NaHCO_3$ of the Ringer solution an equivalent amount of alkali phosphate, no distinct difference could be found, no matter whether this small amount of phosphate was added in the form of primary or secondary phosphate or of a mixture of both. Phosphates at a higher concentration, however, have a decidedly inhibiting effect.

When the blood corpuscles are hemolyzed by dissolving them in distilled water and salt solution is added, such as to give the final mixture the composition of a Ringer solution, no respiration takes place at all either with or without methylene blue, either in the presence or in the absence of sugar, provided the hemolysis is complete. With incomplete hemolysis the result may be intermediary. Hence, the respiration faculty of red blood corpuscles is linked with what is called the structure of the cell just as much as is the glycolytic faculty. None the less, phenyl urethane has no influence on the respiration induced by liver extract.

3. Preliminary Attempts to Characterize or Isolate the Catalyst Supplement

As yet we have not succeeded in isolating the efficient substance from the tissue extract or in separating by any means an active fraction of the extract from an inactive one. The following attempts were made:

a) Heating the extract at 80° destroys the active substance, at least the filtrate of the heated extract is without any effect. When, however the boiled turbid suspension as a whole was used the experiments were obscured by the fact that boiled extracts containing the coagulated particles show a very considerable oxygen consumption themselves which overshadows any oxygen consumption which they might induce.

b) When rat liver is ground in a mortar and extracted with acetone, neither the acetone-insoluble residue nor the residue of the acetone extract showed any effect comparable to that of the fresh tissue extract.

Exposure to light does not influence the effect of tissue extract, no matter whether the respiration takes place in air or in a mixture of 98 per cent CO + 2 per cent O_2. On the other hand, there is a remarkable effect of illumination upon the action of methylene blue. The effect of methylene blue, though it is very marked even in the dark, is strongly increased in the light. This preliminary observation will be elaborated in a subsequent paper.

4. Experimental Part

The oxygen consumption was measured by Warburg's micro-respiration apparatus with Haldane micro-manometer. The volume of the vessels was about 25 cc., and 7 per cent KOH was used as absorbent for CO_2. The temperature was in the majority of the experiments 23°C., in other experiments 37°, and constant within ±.01°. As a rule, rabbit erythrocytes were used, prepared by repeatedly washing citrated blood with the wanted salt solution, and suspending in a volume of the liquid equal to the original volume of the blood sample. In other experiments human erythrocytes were used with the same result. As a rule, fresh blood corpuscles were used. For especial cases, namely, when a strict absence of any trace of sugar was wanted, the corpuscles were used after 24 hours' standing in the ice box. No noticeable difference could be found, as a rule, whether the corpuscles were fresh or 1 day old, disregarding that very slight effect of a residue of glucose which is supposed to be left in fresh corpuscles.

The organ extracts were prepared by grinding the fresh organ in the mortar and gradually adding Ringer solution (or another medium), ten times the weight of the organ. This emulsion was filtered through ordinary filter paper and the turbid liquid free from coarse particles was used. These extracts were always used in fresh condition.

A selection of the experiments is reproduced in the following diagrams. The time "0" in these diagrams, plotted on the abscissa, is usually 20 to 30 minutes after closing the stop-cock of the manometer, because the very first stage sometimes shows irregularities and the linear course of the change of the manometer reading with time is often reached only after this period. From here the linear course is maintained over several hours. The ordinates give the oxygen consumption in cubic millimeters.

Each experimental mixture is composed as follows:

a) 2 cc. of a suspension of blood corpuscles in Ringer solution (containing all corpuscles of 2 cc. pure blood); or, in control experiment, without blood, the equal volume of pure Ringer solution instead.

b) 2 cc. organ extract, or the same volume of pure Ringer solution instead.

c) In the experiments with methylene blue instead of *b*), methylene blue is added to a definite concentration of 0.0006 molarity. (The variation of this concentration has a relatively small effect, down to about a tenfold dilution of the above concentration.)

d) In all experiments containing sugar the concentration is 0.18 per cent in the final mixture.

e) 7 per cent KOH was used as absorbent for CO_2.

SUMMARY

Non-nucleated mammalian erythrocytes do not respire even in the presence of sugar, but they do respire after addition of a small amount of methylene blue.

It is shown in this paper that aqueous extracts of various organs, especially liver, act in the same way as methylene blue. The respiration of erythrocytes induced by an organ extract is not altered in the presence of carbon monoxide.

The content of this respiratory supplement in extracts of organs varies according to the organ: liver and kidney show the best effect; muscle, brain, and blood serum the least.

With hemolyzed erythrocytes no respiration can be induced either by methylene blue or by organ extracts.

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THE KINETICS OF PENETRATION

II. The Penetration of CO_2 into Valonia

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In a previous paper[1] it was shown that in the cells of *Valonia macrophysa* the undissociated CO_2 very soon comes into equilibrium with that in the sea water outside. As this does not tell us in what form CO_2 penetrates[2] it seemed desirable to study the time curves of penetration, for it has been shown[3] that if they follow an equation of the first order the velocity constant, V_M, which is found when molecules alone enter, should theoretically behave like the velocity constant, V_A, found when the ion pair $H^+ + HCO_3^-$ alone enters: furthermore if both molecules and the ion pair $H^+ + HCO_3^-$ enter the velocity constant, V_{MA}, should act like V_M and V_A; but this is not true of V_{Na}, found when only the ion pair $Na^+ + HCO_3^-$ enters, or of V_{MNa}, found when molecules of HA enter in addition to the ion pair $Na^+ + HCO_3^-$. Similarly, if the internal pH value remains constant as the external pH value increases, V_M, V_A, and V_{MA} should, theoretically, remain constant, but V_{Na} and V_{MNa} should increase. If the internal pH value rises, V_M, V_A, and V_{MA} should fall off but V_{Na} should rise: and V_{MNa} should fall or rise, depending on whether molecules or ions penetrate more rapidly.

Furthermore, if we keep the concentration of undissociated CO_2

[1] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, **9**, 255. This paper contains references to previous work on the penetration of weak acids into living cells. For experiments on the penetration of CO_2 into *Valonia* see Brooks, M. M., *Pub. Health Rep.*, 1923, **38**, 1470.

[2] *Cf.* Osterhout, W. J. V., Some aspects of cellular physiology, in Lectures on Plant Pathology and Physiology in Relation to Man, Mayo Foundation Lectures, Philadelphia, 1926–27, p. 179. Also *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 192. A different conclusion was reached in a previous paper (1).

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 261.

constant while increasing that of HCO_3^- and $CO_3^=$ (by raising the pH value and concentration of total[4] CO_2 in the sea water) we should expect an increase in the rate in case HCO_3^- or $CO_3^=$ enters. It was therefore decided to compare the time curves of penetration of CO_2 at high and low pH values, *e.g.*, at pH 4.8 at which the CO_2 present in sea water is considered to be almost completely undissociated, and at pH 6.8 where the dissociation is over 75 per cent.[5]

The experiments were carried out on *Valonia macrophysa* in Bermuda at a temperature of 20° to 24°C.; the temperature did not vary more than 0.5°C. during any one run.

Samples of sea water were prepared in 2-liter Winchester bottles, uniform as to the sea water used, but differing as to content of CO_2: the pH value was either 4.8 or 6.8. All the samples for the series reported here were prepared at one time. They were protected from change in pH value or CO_2 content by being filled without gas space, and having the rubber stoppers wired down. The CO_2 was introduced by adding the required amount of $NaHCO_3$, and the pH value was adjusted by adding 0.1 M NaOH or HCl. The pH value was determined colorimetrically, using the indicators of Clark and Lubs,[6] and Kolthoff's buffers[7] (with allowance for salt error).

The experiments on penetration were carried out in wide-mouthed bottles of about 125 cc. volume. All the bottles required for one run[8] were filled at one time. For this purpose one of the Winchester bottles mentioned above was unstoppered, and a rubber stopper carrying a wide siphon tube was inserted. About 100 cc. of the sea water was withdrawn to wash out the tube, and its place was taken by the same amount of "Nujol" drawn into the Winchester bottle as the sea water ran out. The small bottles were then quickly filled, the end of the siphon tube being allowed to dip below the surface of the entering liquid in order to cut down the loss of gas. Each bottle was stoppered with a rubber stopper as soon as it was filled, without gas space above the liquid. A certain amount of gas escaped from the main volume of sea water as its level fell in the Winchester bottle, but the layer of "Nujol" on top served to reduce this loss.

It is desirable to have cells of uniform size and shape but as this was impossible we endeavored to have the same volume and surface exposed in each case. To get consistent results, the cells must be selected so that the same volume and surface

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[4] By total CO_2 is meant the undissociated and dissociated CO_2 taken together.

[5] *Cf.* 1, p. 259. Also Güntelberg, E., and Schiödt, E., *Z. physik. Chem.*, 1928, **135**, 393.

[6] Clark, W. M., Determination of hydrogen ions, Baltimore, 3rd edition, 1928.

[7] Kolthoff, I. M., and Furman, N. H., Indicators, New York, 1926, pp. 147–48.

[8] By "run" is meant all the separate determinations required for the determination of one time curve at constant pH value and content of CO_2.

are exposed in the determination of each point in a run, and the stirring must be uniform. To achieve the first condition we collected a large number of cells and divided them into four groups of the following approximate volumes, 0.75 to 0.6 cc., 0.5 to 0.4 cc., 0.4 to 0.3 cc., and 0.3 to 0.1 cc. For each small bottle we selected 1 cell from the first group and 2 cells from each of the other groups. And in each of these three groups we chose equal numbers of approximately spherical, and approximately ellipsoidal cells. The total volume of cells in each small bottle was thus about 2.7 cc. The second condition, uniform stirring, was secured by means of a special stirrer (Fig. 1). This consisted simply of a square brass shaft, *A* (Fig. 1), revolving on the bearings *D* and D_1, recessed on opposite sides. The six recesses, *B*, had curvatures which would fit the curvature of the type of bottle used throughout this work. Each recess was provided with a set of two brass rods, *C*, one on each side and so arranged that a bottle

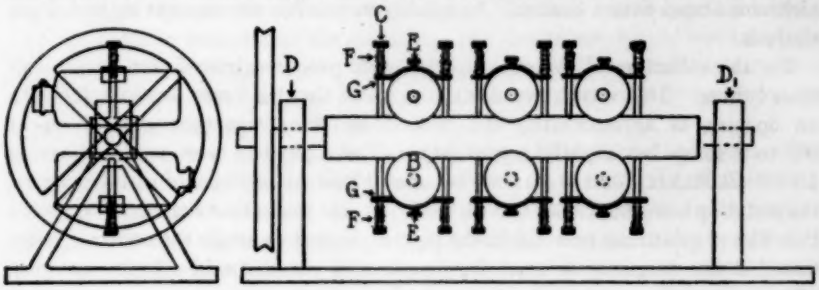

FIG. 1. Diagram of stirrer

could be slipped easily between them (see the lower left recess of the figure). Sliding on each set of rods was a curved brass bar *E* (⅛ inch wide). This was fitted with a handle and it was pressed towards the shaft by means of steel springs *F* coiled around the rods. The bar *E* was prevented from approaching the shaft too closely by the brass pins, *G*, drawn through the rods. With no bottle in place the distance between the center of the bar and the center of the recess was about ¼ inch less than the diameter of a bottle. When it was necessary to insert a bottle the bar could be pulled out slightly by the handle and the bottle put in. On releasing the handle the bar held the bottle firmly against the shaft. It was possible to insert any bottle while the stirrer was in motion. Thus we could start the stirrer and without stopping it determine all the points required for one run. This contributed greatly to the uniformity of stirring throughout a run, and since the shaft was always revolved at the same rate (4 revolutions per minute) the stirring was quite uniform throughout the work. This was about the best rate since the bottles were turned over just fast enough to cause the cells to describe a figure of eight motion in the bottle.[9] This served not only to keep the

[9] That is, across the bottom, then ascending diagonally to the top, across the top, and descending diagonally to the bottom.

cells in motion, but also to keep the CO_2 content of the sea water uniform throughout.

In carrying out an experiment, *i.e.*, the determination of a single point on a time curve, the stopper of a bottle was sharply jerked out (using a file with a sharpened point as a lever), the cells were at once dropped in, the stopper was reinserted tightly without gas space, and the bottle was immediately placed on the stirrer. A small loss of gas was inevitable when the stopper was removed, since there was a momentary vacuum created over the liquid. By jerking the stopper out quickly, this loss was reduced. The whole operation, from the selection of the cells to the commencement of stirring, could be carried out in from 5 to 10 seconds; so that, except in the case of the 1-minute experiments, the proportion of non-uniform stirring was small. About 2 seconds before the end of the experiment the bottle was taken from the stirrer and the cells were poured out onto a nichrome screen over a beaker. As quickly as possible the sap was extracted for analysis.

For the collection of the sap, pipettes were prepared from 5 mm. thick wall glass tubing. This was drawn down to a point about 2.5 mm. in diameter with an opening of approximately 0.5 mm. The point was then ground off at 60° to a sharp but slightly jagged edge. Three pipettes were used, delivering 1.1930 ± 0.0015 cc., 0.8830 ± 0.0008 cc., and 0.8857 ± 0.0010 cc. of distilled water, the last drop being blown out in each case. It was found that the sap always left a thin film of gelatinous material in the pipette, so that to attain the accuracy mentioned it was necessary to treat the pipette with chromic acid solution between fillings.

The extraction of the sap was accomplished by bringing the cell against the sharp edge of the pipette and imparting a twisting motion to it. The saw-like edge instantly cut through the tough cellulose wall, and the sap could be expressed into the pipette by gentle squeezing. As the cell became deflated the protoplasm was detached, and mixed with the sap. To avoid driving this into the pipette the last drop of sap was not extracted. After extraction of the necessary amount of sap, those cells which were not punctured were returned to ordinary sea water for observation of injury and kept under observation for several days:[10] when injury occurred the experiment was rejected.

In sampling, our routine was to proceed from the smallest cells up. An occasional cell softened during the experiment, and as we had no way of determining whether this indicated an increased permeability of the protoplasm we rejected all such cells. Sometimes on attempting to extract the sap from the smaller cells, the rupture spread beyond the rim of the pipette, and part or all of the sap

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[10] The best criterion of injury is microscopic observation (under the high power) which should be frequently carried out during the experiment as reversible injury may occur (*i.e.*, injured spots may appear during the experiment which may become normal in appearance when the cell is returned to its normal environment).

was lost. For these reasons uniformity in the size of the cells taken for analysis was not attained.

Immediately after extraction the sap was transferred to a Van Slyke constant pressure carbon dioxide apparatus, and the analysis was carried out according to the method of Van Slyke.[11] In every case (regardless of whether the sample was slightly greater or less than 1 cc.) the total volume was made up to 2.5 cc. so that the tables given in the paper quoted could be used.

The accuracy of the analysis for CO_2 by the Van Slyke method can be calculated from the figures given by Van Slyke in the paper quoted above. The probable error of the mean is about 0.17 per cent of the mean for a concentration range comparable with that of our solutions. This error is assumed to include the error involved in measuring out the solution by pipette. The error produced by the loss of gas from the sap during transfer to the Van Slyke apparatus may be more serious than the formal error of analysis. But it is certainly small in comparison with the variation of the material. For this reason we did not adopt the method of transfer suggested by Van Slyke and Neill,[12] by which the sample is delivered directly into the gas burette. Instead we kept this error as low as possible by transferring the sap rapidly.

A further source of error was the variation of the CO_2 content of the sea water in a single run. This was unavoidable in our method of filling the small bottles. The possibility of carrying out this operation by causing the sea water to displace an oil like "Nujol" upwards, or mercury downwards, was rejected because the oil could not be displaced cleanly and the cells became coated with grease, while mercury was rejected because of the toxic nature of its salts. To evaluate this error we filled a number of bottles with CO_2-containing sea water at pH 4.8, in the usual manner, and then unstoppered them at intervals during 2 hours, and analyzed for CO_2. The probable deviation from the mean was found to be 0.007 cc. or 1.1 per cent.

Unless otherwise stated the curves are drawn free-hand to give a rough fit.

The data for four time curves at pH 4.8 and four at pH 6.8 are given[13] in Table I.

[11] Van Slyke, D. D., *J. Biol. Chem.*, 1917, **30**, 347.

[12] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[13] It may be of interest to compare the figures for penetration of CO_2 into living *Valonia* with those obtained by Northrop (Northrop, J. H., *J. Gen. Physiol.*, 1928–29, **12**, 435, Table III) for penetration through a (previously well dried) collodion membrane separating a solution of NaOH from a solution of CO_2. Since in his experiments CO_2 was dissolved in distilled water we may use for comparison our results at pH 4.8. If we consider that 0.1 mg. CO_2 penetrates in 1 minute per cubic centimeter of sap at 20°C. when the external concentration is 0.904 mg. CO_2 per cubic centimeter sea water the pressure at the start is 0.5 atmosphere (allowing 0.009 mg. CO_2 per cubic centimeter sap at the start) so that

If the penetration is a simple diffusion we might expect the time curve to obey an equation of the first order. The velocity constants calculated from the equation,[14]

$$K = \frac{1}{t} \log \frac{a}{a - x}$$

are shown in Fig. 2. Since they fall off from the start it might seem that the equation for a dimolecular reaction might give better values: accordingly calculations were made with the dimolecular equation

$$K_1 = \frac{1}{t(a - b)} \log \frac{(a - x)b}{(b - x)a}$$

putting $a = 1.00$ and $b = 1.35$. Since this gives a more constant value of K_1 (as shown in Fig. 2) it might seem that the dimolecular equation should be employed, and that possibly a reaction occurs between the penetrating substance and some constituent of the protoplasm. We find, however, that when we multiply all the ordinates of a time curve by the same factor (varying the factor from curve to curve) so as to make the equilibrium value the same in all cases, the curves agree within the limits of experimental error (Fig. 3). Hence the times for half completion must be approximately the same in all cases. This would not be the case if they obeyed a dimolecular equation[15] (unless

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at 1 atmosphere 0.2 mg. CO_2 would penetrate per minute per cubic centimeter of sap and for a cell containing 0.41 cc. the amount penetrating would be 0.2 (0.41) = 0.082 mg. Such a cell may be regarded as having a surface of 2.8 sq. cm. If the surface were 1 sq. cm. the amount penetrating per minute would be 0.082 ÷ 2.8 = 0.0292 mg. and the amount penetrating per day would be 42 mg. = 21.2 cc. The figure given by Northrop is 8.6×10^{-4} cc. per square centimeter per day for a membrane 1 cm. thick. If we consider the protoplasm to be in the neighborhood of 0.001 cm. thick this would give 0.86 cc. per day for collodion but as the non-aqueous part of the protoplasm is undoubtedly much thinner this figure is probably much too small. It would seem therefore that penetration may be of the same order of magnitude for such collodion membranes and for *Valonia*.

[14] Common logarithms are used for convenience and a is put equal to the final equilibrium value of the time curve: this value corresponds approximately to that of the undissociated CO_2 in the sea water but is not equal owing to differences in solubility. (*Cf.* 1.)

[15] Another way of looking at the matter is to say that if the curves agree when multiplied up (so as to have the same equilibrium value) the time for half com-

TABLE I

Increase of Total CO_2 (Observed Value Less the Amount Present at the Start) in Cells Unlike in Size and Shape. Each Figure Represents the Average of 20 or More Cells

Time	pH of sea water 6.8				pH of sea water 4.8			
	mg. of total CO_2 per cc. of sap				mg. of total CO_2 per cc. of sap			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4
min.								
1	0.035	0.044	0.044	0.062	0.100	0.149	0.071	0.054
3	0.094	0.134	0.095	0.115	0.210	0.243	0.200	0.180
5	0.147	0.225	0.146	0.143	0.315	0.404	0.231	0.271
7	0.163	0.254	0.204		0.494	0.560	0.304	0.317
9	0.186	0.317	0.217		0.541	0.583	0.359	0.328
12	0.224	0.397	0.240	0.266	0.603	0.696	0.403	0.441
15	0.242	0.433	0.258	0.302	0.651	0.766	0.480	0.533
20	0.269	0.527	0.302	0.307	0.719	0.874	0.545	0.631
30	0.302	0.569	0.391	0.378	0.833	0.969	0.645	0.748
45	0.353	0.601	0.439	0.408	0.862	1.016	0.712	0.811
60	0.396*	0.649*	0.452	0.440	0.954	1.116*	0.791	0.891
120	0.395	0.643	0.482	0.456	0.990	1.049	0.791	0.891

* This is taken as the equilibrium value.

pletion must be the same for all as is the case when they are of the first order and all have the same velocity constant. But if they are of the second order this is not true. For example, if the two reactants are equal at the start and if the velocity constant is 1 we have as the time for half completion, t, the following:

$$t = \frac{x}{a(a - x)}$$

where a is the original concentration of each reactant or the final equilibrium value of x (x being the amount of substance formed by the reaction). Since $x = 0.5\ a$ at half completion, we have

$$t = \frac{0.5a}{a^2 - 0.5a^2} = \frac{1}{a}$$

that is, t is inversely proportional to a. Hence if with different concentrations of reactants the curves have the same velocity constant, it follows that the times for half completion cannot be the same and the curves will not be identical when the equilibrium values are made equal by multiplying the ordinates.

If the reaction is dimolecular we should expect the velocity constant to increase

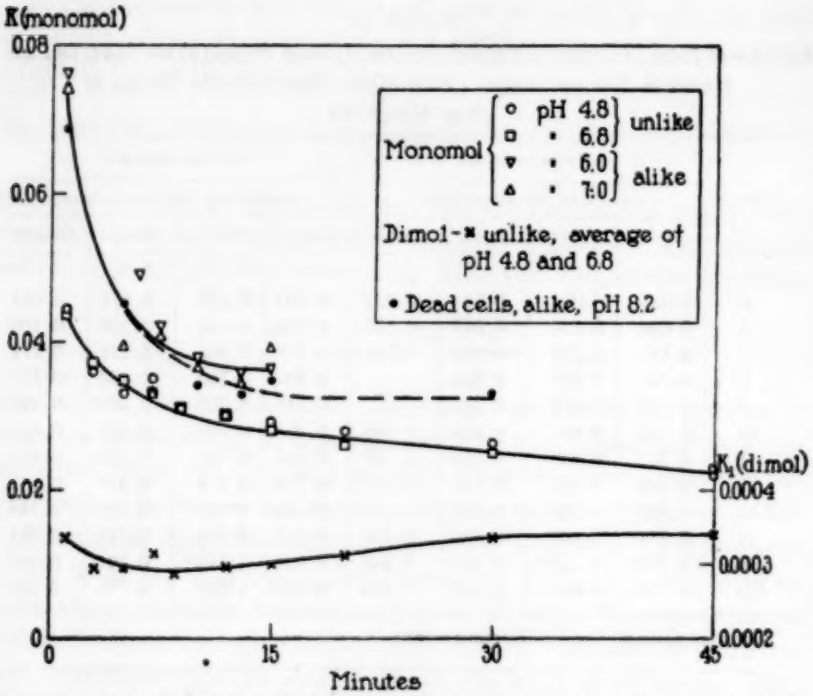

FIG. 2. Monomolecular velocity constants for living and dead cells calculated from the equation $K = \frac{1}{t}\log\frac{a}{a-x}$, where x is the increase of total CO_2 in the sap and $a = 1.00$, the equilibrium value: also dimolecular constants for living cells calculated from the equation $K_2 = \frac{1}{t(a-b)}\log\frac{(a-x)b}{(b-x)a}$, where $a = 1.00$, $b = 1.35$.

as the external concentration of CO_2 increases, but Fig. 3 shows that this is not the case (for example, when we compare experiments at the same external pH value).

If a compound is formed between the protoplasm and CO_2, we should expect that in the case of (H^+, HCO_3^-) ion pairs we should have to deal with two consecutive dimolecular reactions: $H^+ + HCO_3^- \rightarrow H_2CO_3$ at the surface, followed by H_2CO_3 + protoplasm $\rightarrow H_2CO_3$ protoplasm, and even a termolecular reaction: $H^+ + HCO_3^-$ + protoplasm $\rightarrow H_2CO_3$ protoplasm. In either case the imposition of the second reaction on the reaction where molecules alone are involved ought to alter the form of the curve. The fact that it does not might be regarded as evidence for the view that (H^+, HCO_3^-) ion pairs do not penetrate. And as we pointed out above there is also evidence for the view that (Na^+, HCO_3^-) ion pairs do not penetrate.

their velocity constants happened to fall in just the right relation to the concentrations employed: this would require an extraordinary set of coincidences) but would if they followed the monomolecular formula. The question arises, How can they follow this formula

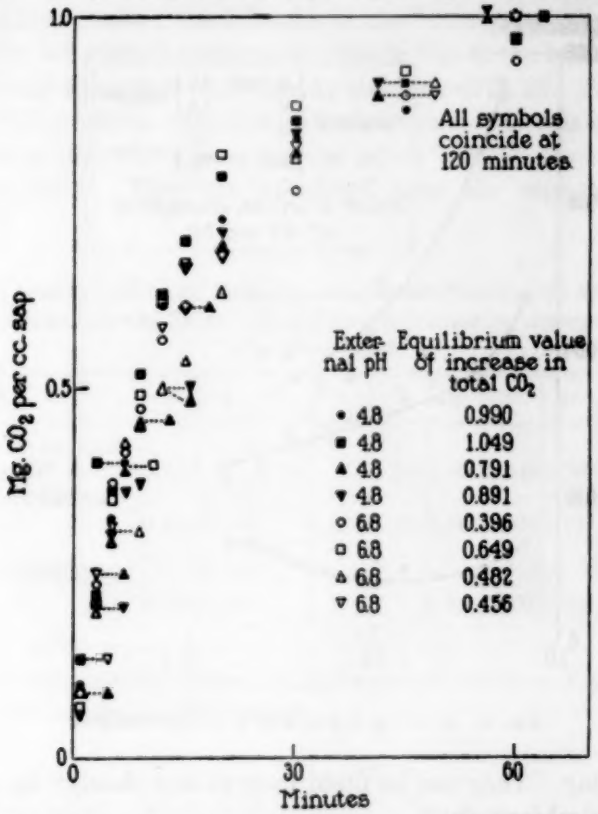

FIG. 3. Shows the result of bringing all the curves (for unlike cells) to the same final value by multiplying all the ordinates of a curve by the same factor (using a different factor for each curve).

To avoid crowding some symbols have been displaced to one side but these are connected by a line to the point where they belong.

when the velocity constants fall off as in Fig. 2? Now in the case of dead cells,[16] where there would seem to be no question that the ordinary

[16] These often show a slightly higher velocity constant at pH 4.8 than at pH 6.8 which is to be expected since the diffusion constant of CO_2 is greater than that

diffusion formula is followed, the constants often fall off in much the same way. It would therefore seem possible to regard the curves as of the first order but having a "velocity constant" which falls off from the first as would be the case, for example, if the temperature

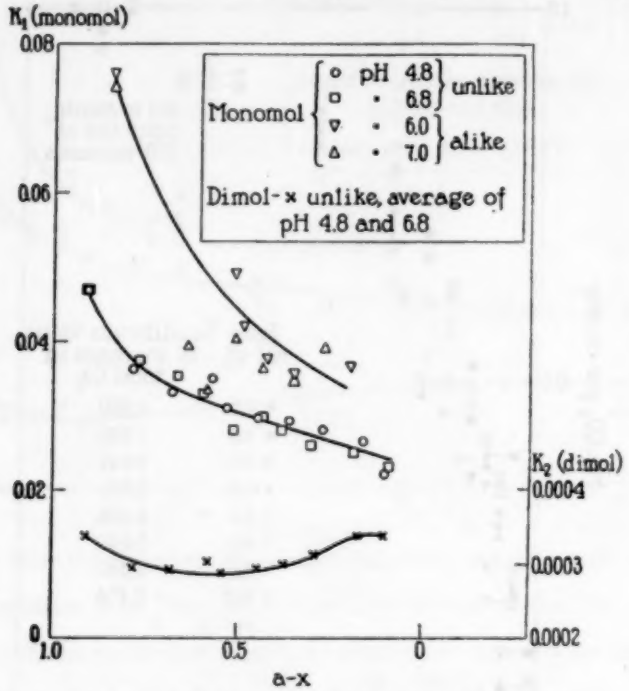

FIG. 4. As in Fig. 3, but with $a - x$ as abscissae

were falling. They can be fitted more or less closely[17] by means of the empirical formulae[18]

$$K_1 = \frac{ba}{tx} \ln_e \frac{a}{a-x} - x \text{ and } K_2 = \frac{bx^n}{t} \ln_e \frac{a}{a-x}$$

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of Na_2CO_3 (*cf.* Internat. Critical Tables, V, 63, 67) and the cell wall is so permeable that only ordinary diffusion would appear to be involved. We might apply here the equations employed elsewhere[3] but this is not necessary since no protoplasm is present and consequently there is only one phase (the cell wall being so permeable that it need not be regarded as a distinct phase).

[17] These may fit the latter part of the curve better than the earlier part.

[18] In these formulae K_2 is not independent of a.

other empirical formulae may be used in similar cases, *e.g.*,

$$K_2 = \frac{1}{t} \ln_e \frac{a}{a - bx^n} \text{ and } K_2 = \frac{1}{t} \frac{ab}{x^n} \ln_e \frac{a}{a - x}$$

Let us now consider the behavior of the "constants" more closely. When they are plotted against $a - x$ (as in Fig. 4) the curve does not differ greatly from that with time as abscissae (Fig. 2). It is better to plot the constants calculated for short time intervals at various points along the curve: these may be called "momentary constants" for convenience. They are calculated from the smoothed curve

TABLE II

Increase of Total CO_2 (Observed Value Less the Amount Present at the Start) in Cells Alike in Size and Shape. Each Figure Represents the Average of 20 or More Cells

Time in minutes.....		1	5	6	7	7.5	10	13	15	30	120
Live cells	pH of sea water = 6	0.159		0.489		0.510	0.573	0.649	0.709		0.990
	pH of sea water = 7	0.073	0.171		0.193	0.236	0.266	0.305	0.337		0.469
Dead cells	pH of sea water = 8.2	0.181	0.501				0.668	0.777	0.844	1.112	1.240

(*A*, Fig. 5) obtained by averaging all the points shown in Fig. 3. For this purpose we employ the formula

$$K_m = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$$

where x_1 is the total CO_2 penetrating in the time t_1 and x_2 is that penetrating in the time[19] t_2. The results of these calculations, shown in Figs. 6 and 7, offer nothing essentially new.

It is therefore clear that the constants really fall off and it might be natural to ascribe this to differences in size and shape as the result of which some cells would have a relatively greater surface and

[19] In the first part of the curve the interval between t_1 and t_2 is in seconds but in the latter longer intervals are used.

consequently reach equilibrium sooner than the others so that when the whole group is considered penetration appears relatively rapid at the start.

To test this suggestion would require a quantity of cells uniform in size and shape which there seemed to be no immediate prospect of obtaining. Eventually, however, a supply was secured and the experiments were carried out. The results obtained are shown in Table II and Fig. 5; the velocity "constants" fall off as is shown in Figs. 2, 4, 6, and 7.

The experiments were carried out as before except that a Van Slyke constant volume apparatus was used and in order to avoid loss of CO_2 higher pH values (6 and 7) were used. The temperature varied between 18° and 20°C.

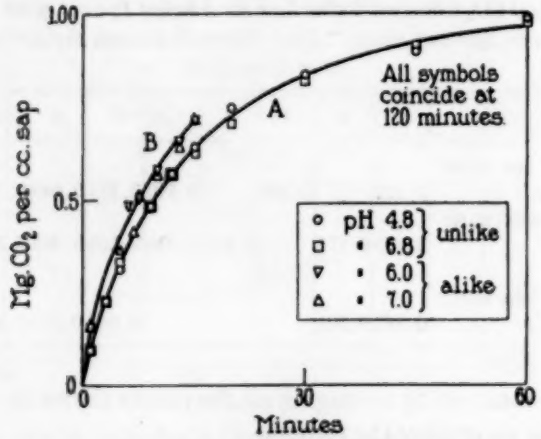

FIG. 5. Time curves showing increase of total CO_2 in the sap of living cells, (*A*) unlike in size and shape, (*B*) similar in size and shape. The ordinates have been multiplied to make the equilibrium value 1.0 in each case.

It is evident that if some of the cells were more permeable than others the effect would be the same as if their surface were relatively large and this would cause a falling off of the "constants." We are inclined to accept this explanation.

Experiments on dead cells bear out this idea and are in harmony with investigations on the penetration of KIO_3 made several years ago by Mr. W. C. Cooper, Jr. (in collaboration with the senior author). Studying the penetration of KIO_3 into dead cells of the same size and

shape it was found that the velocity "constants" sometimes fell off and sometimes remained practically constant. The latter condition was favored by using cells whose permeability was similar because they were freshly killed. When such cells were mixed with others that had stood for a long time in sea water after killing (so that their walls were partially disintegrated) the constants fell off much more

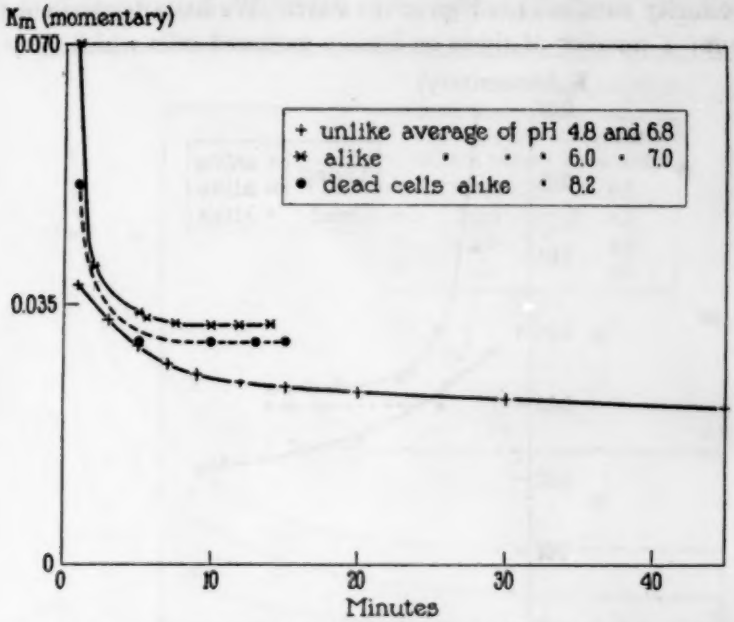

FIG. 6. "Momentary" constants calculated for short intervals along the time curves from the formula $K_m = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$, where $t_2 - t_1$ represents a very short time interval, x_1 is the increase in total CO_2 in the sap at the time t_1, x_2 the increase at the time t_2, and a the equilibrium value.

markedly. This was doubtless due to the fact that some of the dead cells were more permeable than the others.

Since under the most favorable conditions the velocity constants do not fall off when dead cells of the same size and shape are employed, we are inclined to attribute the falling off with cells of the same size and shape, whether alive or dead (for dead cells[20] see Table II and

[20] These experiments were carried out with sea water at pH 8.2.

Figs. 2, 6, and 7), to differences in permeability which cause a more rapid penetration on the part of certain cells: for when these have reached equilibrium penetration continues in the others so that the process for the whole group seems relatively rapid at the start.[21]

There is always a small amount of CO_2 present in the sap even before its exposure to the CO_2-bearing sea water, which would tend to make the velocity constant too high at the start. We have determined this amount a number of times on freshly gathered cells which may be

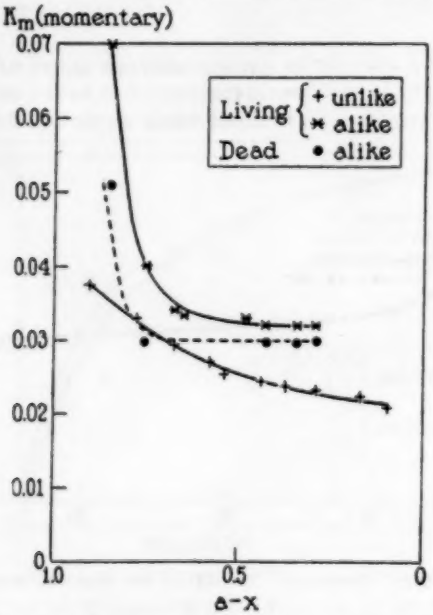

FIG. 7. As in Fig. 6, but with $a - x$ as abscissae

assumed to be growing, and on cells which have been kept in the laboratory and have practically ceased to grow. Very little difference was found between the two groups and the average amount was 0.009 mg. of CO_2 per cubic centimeter of sap. This was subtracted from the observed figures before calculating the velocity constants. It may

[21] It might be thought that if there is much difference in permeability an occasional sample would consist largely of cells of high or of low permeability. The experiments with cells of the same size and shape are not extensive enough to settle this point: the figures for one set of determinations are given in Table III.

be added that the production of CO_2 during the experiment is too small to affect the result.

Another factor which might affect the velocity of penetration is the change in the pH of the sap as the CO_2 concentration increases. We have investigated this change experimentally. Fig. 8 gives our results. Our experiments were not exhaustive but it seems safe to say that the rate of change in pH of the sap is dependent chiefly on the rate of

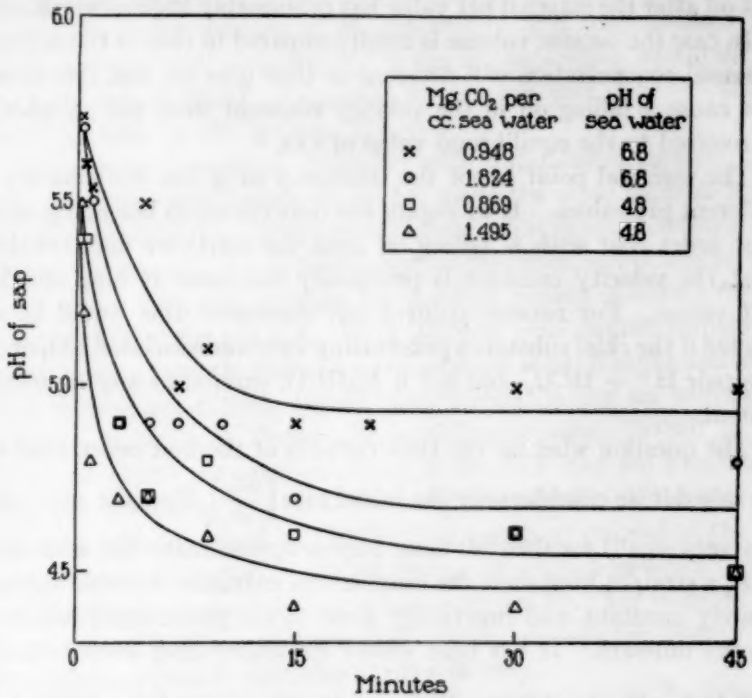

FIG. 8. Changes in the pH value of the sap during the entrance of CO_2

accumulation of CO_2 in the sap, and that it is independent of the pH of the sea water, except as a lower pH of the sea water means that a greater proportion of CO_2 is in molecular form. Assuming that when the pH of the sap has fallen to 4.7 the CO_2 is present almost entirely as molecules, we see that this condition of association may be reached early in the runs (and might be reached earlier in the protoplasm than in the sap). We are inclined to think that this means that there

is little or no penetration of the ion pair $Na^+ + HCO_3^-$ for the entrance of $NaHCO_3$ would raise the pH, opposing the drop produced by the entrance of molecules of CO_2.

If it is chiefly the molecules which enter, the decrease of internal pH value during penetration would tend to make the velocity constant appear greater[3] and greater as long as it continued, but as a matter of fact, the value of K is found to fall off at the start and it continues to fall off after the internal pH value has presumably become constant.[22]

In case the outside volume is small compared to that of the cells the external concentration will decrease as time goes on, but this should not cause a falling off in the velocity constant since the calculation is referred to the equilibrium value of CO_2.[23]

The essential point is not the constancy of K but its behavior at different pH values. If we regard the time curves as belonging to the first order (but with K falling off from the start) we may conclude that the velocity constant is practically the same at high and low pH values. For reasons pointed out elsewhere[3] this would be expected if the chief substance penetrating were undissociated CO_2 or the ion pair $H^+ + HCO_3^-$, but not if $NaHCO_3$ entered to any noticeable extent.

The question whether the time curve is of the first order need not be raised if we consider only the initial rate $\left(\frac{dS_i}{dt}\right)_0$ (*i.e.*, the rate when t is very small) for then all time curves approximate the zero order (*i.e.*, a straight line) since the outside concentration remains approximately constant and practically none of the penetrating substance moves outward. It has been shown elsewhere[3] that when both un-

[22] As the pH value of the sap decreases the per cent of loss during manipulation increases because more of the CO_2 is in volatile form but this would not cause a falling off in the velocity constant because it affects the equilibrium value even more than the early values.

[23] The case is similar to that of a reversible monomolecular reaction $A \rightleftharpoons B$ where A decreases but the time curve of increase of B is monomolecular (*cf.* Mellor, J. W., Higher mathematics for students of chemistry and physics, London 1922, p. 228) when the calculation is based on the equilibrium value.

In these experiments the volume of cells was relatively small in comparison with that of the sea water and in no case did the external concentration of total CO_2 fall off more than 2 per cent during an experiment.

dissociated molecules and the ion pairs $H^+ + HCO_3^-$, $Na^+ + HCO_3^-$ and $K^+ + HCO_3^-$ enter we may write

$$\left(\frac{dS_i}{dt}\right)_b = P_M M_o + P_A H_o A_o + P_{NaA} Na_o A_o + P_{KA} K_o A_o$$

where M_o is the molar concentration of undissociated CO_2 outside (including H_2CO_3), H_o the external concentration of H^+, Na_o that of Na^+, K_o that of K^+, and A_o that of HCO_3^-; S_i is the total CO_2 inside, P_M is the amount of M_o entering in unit time through unit surface under unit pressure, P_A is the corresponding value for the ion pair $H^+ + HCO_3^-$, P_{NaA} that for the ion pair $Na^+ + HCO_3^-$, and P_{KA} that for the ion pair $K^+ + A^-$.

The results here given (together with unpublished figures) show that when the equilibrium values are made the same by multiplying the ordinates the time curves agree closely. For example, when all the curves are brought (by multiplication of the ordinates) to the equilibrium value 1.00, the average for the first minute is 0.0965 for pH 4.8 and 0.0960 for pH 6.8. With uniform cells (which furnish the most decisive test) the values for the increase at pH 6 at the end of the first minute are 0.1568, 0.1582, and 0.1605, giving an average of 0.1585. In order to bring the equilibrium value (0.990) to the standard (1.00) we must multiply it by 1.01 and we accordingly multiply 0.1585 by the same factor giving 0.160. How does this compare with the result at pH 7? The values at the end of the first minute are 0.0728, 0.0724, 0.0738, giving an average of 0.073. Since the equilibrium value 0.469 must be multiplied by 2.132 to bring it to the standard (1.00) we multiply 0.073 by the same factor, giving 0.156 which is very close to the value at pH 6 (0.160).

It would seem that the value of $\left(\frac{dS_i}{dt}\right)_b$ is practically the same at low and at high pH values. As the equilibrium values are the same the values of M_o must also be the same (provided the internal pH values are approximately the same, as seems to be the case). Now at pH 4.8 the concentration of HCO_3^- approaches zero so that the value of $P_A H_o A_o + P_{NaA} Na_o A_o + P_{KA} K_o A_o$ reduces almost to zero and when $\left(\frac{dS_i}{dt}\right)_b$ and M_o have the same values at pH 6.8 and at 4.8 the value of

the expression $P_A H_o A_o + P_{Na} Na_o A_o + P_{KA} K_o A_o$ must be nearly zero at pH 6.8 even though the concentration of HCO_3 and of A_o is relatively large. This can only mean that there is little penetration of the ion pairs $Na^+ + HCO_3^-$ and $K^+ + HCO_3^-$.

In other words, the results indicate that adding $NaHCO_3$ to the external solution has little effect on the rate of penetration provided we do not at the same time change the external concentration of undissociated CO_2 so that we conclude that there is very little penetration of HCO_3^- or $CO_3^=$.

It may be objected that since the value of $\left(\frac{dS_i}{dt}\right)_b$ is not directly

TABLE III

Increase of Total CO_2 (Observed Value Less the Amount Present at the Start) in Cells Alike in Size and Shape at the End of 2 Minutes. Each Figure Represents the Average of 10 or More Cells

pH of sea water	mg. of total CO_2 per cc. of sap						
	Individual experiments						Average
6	0.0111	0.0161	0.0070	0.0130	0.0305	0.0073	0.0142
7	0.0130	0.0146	0.0071	0.0124	0.0297	0.0067	0.0139

Differences between the columns are due to differences in the concentration of CO_2 in the sea water in the different experiments but in each column the concentration of undissociated CO_2 in the sea water was the same for both pH values.

determined there may be some uncertainty in this procedure. We have tested this matter experimentally in the following way.

Two samples of sea water, one at pH 6 and the other at pH 7, containing approximately the same amount of free CO_2 were prepared and placed in 125-cc. bottles. Then by means of an apparatus similar to that used by Osterhout and Dorcas[1] gas was circulated between the two solutions for 2 hours, at the end of which time it was assumed that the CO_2 tension was the same in each. The two solutions were then used in penetration experiments on cells of about the same size and shape. The time of exposure was 2 minutes. The results of six pairs of determinations are given in Table III.

It will be seen that the amount of CO_2 penetrating during the 2 minutes of exposure in these experiments is much smaller than that penetrating in the same time in the experiments reported above. The reason for this is that the smaller amounts of CO_2 can be distributed equally between the two samples of sea water

in a reasonable time, while at the same time the losses due to diffusion through the rubber part of the distributing system, and during the penetration experiments are very much cut down.

In spite of the small amount of CO_2, by reducing the gas to the smallest volume permitted by the Van Slyke constant volume apparatus, we have measured it with an error which does not exceed twice the error of the previous measurements.

Table III shows that the average values of 6 pairs of experiments check within 2 per cent which is well within the experimental error which we believe to be not greater than 6 per cent. It would therefore seem that the rate of penetration depends only on the concentration of undissociated CO_2 and not on that of HCO_3^- or $CO_3^=$ since in these experiments the concentration of ions could be greatly varied without affecting the rate of penetration as long as the concentration of undissociated CO_2 remained unaltered.

SUMMARY

The rate of penetration of CO_2 into living cells of *Valonia* has been studied at high and low pH values.

The time curve of penetration appears to be of the first order but with a "velocity constant" which falls off from the start.

The evidence indicates little penetration of ions. This is shown by (*a*) the similarity of velocity "constants" at high and low pH values, (*b*) the rate of penetration, which remains constant as long as the external concentration of undissociated CO_2 remains constant no matter how much the concentration of ions varies.

CALCULATIONS OF BIOELECTRIC POTENTIALS

I. Effects of KCl and NaCl on Nitella

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In this paper the chemical and concentration effects of KCl and NaCl in *Nitella* (Table I) are examined with a view to mathematical treatment.

TABLE I

Chemical and Concentration Effects in Nitella

0.001 M KCl positive* to 0.01 M KCl	54.7 mv.**
0.001 M NaCl positive to 0.01 M NaCl	20.9 mv.***
0.01 M NaCl positive to 0.01 M KCl	82.9 mv. †
0.01 M NaCl positive to 0.005 M KCl + 0.005 M NaCl	68.8 mv. ‡

* *I.e.*, positive in the external circuit.

** Average of 25 experiments: probable error of the mean 1.6 % of the mean.

*** " " 23 " " " " " " 4.5 % " " "

† " " 8 " " " " " " 3.2 % " " "

‡ " " 16 " " " " " " 3.8 % " " "

The experiments were performed on *Nitella flexilis* at a temperature of 19° to 20°C. according to the technique previously described.[1]

The concentration effects were measured in the best range (0.01 M to 0.001 M[1]) on intact cells, giving values higher than those previously obtained[1] for KCl with intact cells (and more nearly like those obtained with cells having one spot killed with chloroform[2]); this is probably due to the fact that in the present experiments broader contacts were employed, reducing the protoplasmic resistance, which, as

[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761; *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 838.

[2] When one spot is killed with chloroform the resistance which is called R_3 in the subsequent discussion is reduced: in the present experiments R_3 was reduced by broad contacts (without applying chloroform).

will be explained presently, gives a more accurate measure of the P.D. In addition broader air gaps were used, thus giving better values. Also the intact cells appear to have been in better condition than in the previous experiment (it is well known that injury causes the values to fall off). During the earlier experiment the criteria of injury and the methods of preparing the cells were not nearly so good as now. Furthermore, different concentrations of KCl were placed in succession on the same spot but in the previous experiment they were applied simultaneously to different spots.

I wish to thank Mr. Philip R. Averell for the care which he has bestowed upon these experiments.

Following the conceptions developed in previous papers we may picture the protoplasm as shown in Fig. 1. The potential at the outer surface of X may be called E_{AX}, the corresponding value at C being

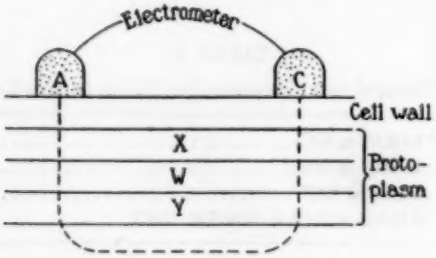


FIG. 1. Hypothetical diagram of the protoplasm of *Nitella*. The principal circuit is shown by the broken line.

E_{CX}. The sum of the other potentials at A may be called E_A, the corresponding value at C being called E_C. The total E.M.F. (which we may call E) is $E = E_A + E_{AX} + E_C + E_{CX}$. Since ordinarily E_A and E_C are equal and opposite they cancel out and we may put $E = E_{AX} + E_{CX}$.

The observed P.D. may be due to potentials of various kinds. The Donnan potential will be left out of account because the cell cannot very well be in equilibrium with two different solutions at the same time, and it does not seem probable that a local pseudoequilibrium can be set up in a few seconds at A or C between the aqueous layer,[3] W, of the protoplasm and the external solution (the case being en-

[3] To set up a new equilibrium in W would involve a new equilibrium between W and the sap.

tirely different from establishing a pseudoequilibrium between the outer surface of X and the external solution as discussed later on). If the system is on the way to a Donnan potential the P.D. will depend largely on mobilities. We shall therefore consider only diffusion

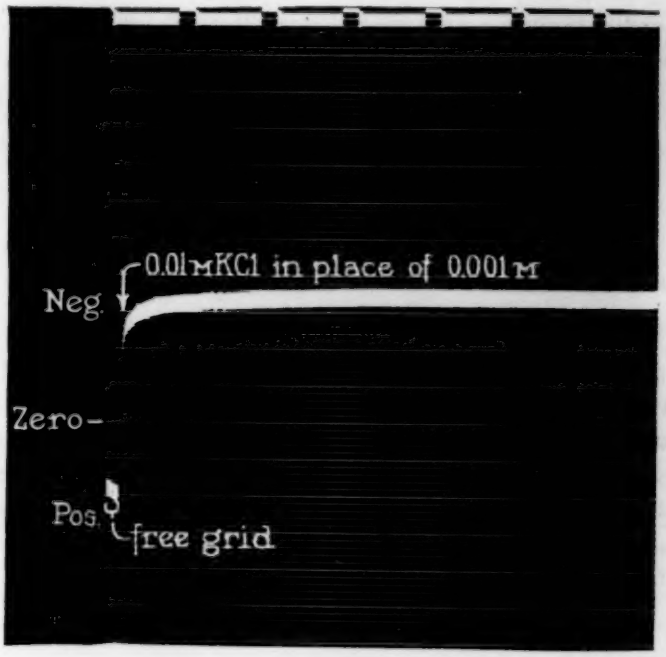


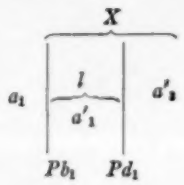
FIG. 2. Photographic record showing the effect of substituting 0.01 M KCl for 0.001 M KCl at A (Fig. 1) when C is in contact with 0.001 M KCl. Before the application of 0.01 M KCl at A the recording instrument is connected to C and to a cup containing 0.01 M KCl but the cell does not touch this cup (this part of the record is marked "free grid"). The left-hand end of the cell (A), which is imbibed with 0.001 M KCl, is wiped off and is then dropped into the cup and at the moment of contact the curve begins to rise: equilibrium is quickly established. The time marks represent 5-second intervals.

potentials and phase boundary potentials. Let us begin with calculations on the basis of diffusion potentials alone.

Calculation of Diffusion Potentials

If we apply 0.001 M KCl at A we may suppose that its behavior at the outer surface of X is like that with non-aqueous substances in

general: it has been found that the P.D. set up with such substances reaches its full value very quickly and this seems to be also the case with protoplasm as shown in Fig. 2. The usual assumption is that the outer layer of the non-aqueous substance (which may be only a few molecules thick and which may be called l) very quickly comes into pseudoequilibrium with the aqueous solution. If this be the case and there is little or no phase boundary potential the mean ionic activity ($a_1 = \sqrt{a_K a_{Cl}}$) of KCl in the aqueous solution must be equal to that in X which is $a'_1 = \sqrt{a'_K a'_{Cl}}$. We then have the system

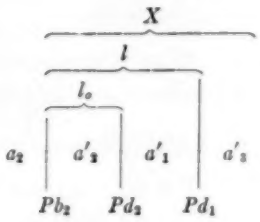


where a'_3 represents the mean ionic activities of all the salts in X where it is in equilibrium with tap water (in which the cell has been kept previous to the application of KCl), Pb_1 is the phase boundary potential and Pd_1 is the diffusion potential. The resulting P.D. in the outer portion of X at A may be called $E_{AX\ 0.001}$ and that at C may be called $E_{CX\ 0.001}$.

We may then write

$$E_{AX\ 0.001} = E_{CX\ 0.001} = Pb_1 + Pd_1$$

If we allow the solution of KCl 0.001 M to act for a few minutes, until the layer l has reached appreciable thickness, and then apply KCl 0.01 M we may conclude from the photographic records (which resemble Fig. 2) that in a very few seconds the outer part of l, which we may call l_o (consisting of only two or three layers of molecules), comes into pseudoequilibrium with the external solution so that we have the system



where a_2 is the mean ionic activity of KCl in the aqueous solution, a'_2 its mean ionic activity in l_o, and Pb_2 the phase boundary potential. The resulting P.D. may be called $E_{AX\ 0.01-0.001}$ so that we may write

$$E_{AX\ 0.01\text{-}0.001} = Pb_2 + Pd_2 + Pd_1$$

In order to obtain this we first measure the P.D. with 0.001 M KCl at both A and C (this is ordinarily not far from zero, otherwise the experiment is rejected). We leave these solutions in place for several minutes[4] and then replace the 0.001 M KCl at A by 0.01 M KCl and measure the P.D. immediately[5] thus obtaining the value of $E_{AX\ 0.01-0.001} - E_{AX\ 0.001}$ (concentration effect) which may be called $E_{conc.}$ so that we may write

$$\begin{aligned} E_{conc.} &= E_{AX\ 0.01\text{-}0.001} - E_{AX\ 0.001} \\ &= Pb_2 + Pd_2 + Pd_1 - (Pb_1 + Pd_1) \\ &= Pb_2 - Pb_1 + Pd_2 \end{aligned}$$

If the phase boundary potentials Pb_1 and Pb_2 are approximately equal we may put

$$E_{conc.} = Pd_2$$

It seems probable that electrical conditions in the protoplasm may correspond to some extent with the diagram in Fig. 3 (the circuits are, of course, not insulated from each other in the protoplasm). Here I_1 represents the current in the cell wall, I_2 that in X, I_3 that in Y and sap. E_1 is a small diffusion potential in a "horizontal" direction in the cell wall due to concentration effect (this is absent when we deal with chemical effects): E_2 is the diffusion potential in a "horizontal" direction in X and E_3 the potential in a "vertical" direction in X (the terms "horizontal" and "vertical" presuppose that the cell is oriented as in Fig. 1), R_1 is the "horizontal" resistance of the cell wall, R_2 the "horizontal" resistance of X, and R_3 the "vertical" resistance of X and Y at A and C plus the "horizontal" resistance of the sap.

Assuming that E_3 corresponds to what we have called $E_{conc.}$ above it is evident that we approach its true value when we make R_1 large and R_3 small. We make R_1 large by increasing the distance between the contacts (this was 2 to 5 cm.) and diminish R_3 by making the contacts broad (in this case 2 cm.): R_3 is often reduced by action currents (due to handling or to applications of solutions at a dis-

[4] The P.D.'s usually remain quite constant during this interval.

[5] The measurements are made from photographic records similar to that shown in Fig. 2.

tance from the contacts) which have been shown by Blinks[6] to reduce the protoplasmic resistance. When the wall is imbibed with tap water as in most of these experiments the values, according to Blinks,[6] are approximately as follows: R_1 = 10 to 25 megohms, probably R_2 = 100 megohms or more, R_3 = 0.4 to 0.6 megohms. We assume that E_1 corresponds to the concentration effect due to the cell wall[1] which acts very much like additional resistance in R_1 so that the greater E_1 (provided it does not exceed E_3) the nearer we come to[7] the true value of E_3 (for 0.01 *vs.* 0.001 M KCl the value of E_1 is about 17 mv.). Under these circumstances we measure practically the full value of E_3: this is apparent on setting up the usual Kirchoff equations (P.D. $= E_1 + I_1R_1 = E_2 - I_2R_2 = E_3 - I_3R_3$ and $I_1 = I_2 + I_3$) and is indicated experimentally by the near approach of the observed concentra-

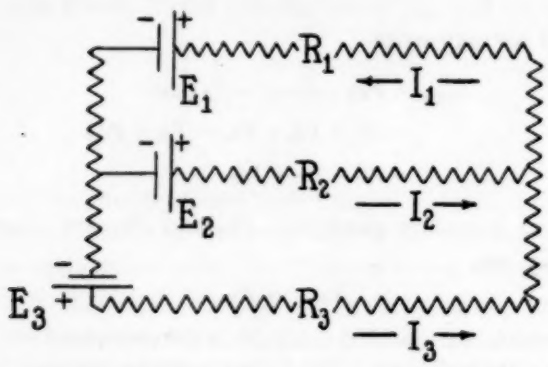


FIG. 3. Hypothetical diagram of electrical conditions in *Nitella*. See text.

tion effect of 0.01 *vs.* 0.001 M KCl (54.7 mv.) to the theoretical maximum (56.215 allowing for activities).

The most reliable measurements are obtained when the cells are in the best condition and fairly free from action currents, since the latter often produce an effect analogous to that of an outward movement of K^+ from the sap[6] to the outside of the protoplasm. It is evident that if such a movement should occur when measuring the P.D. of 0.001 M KCl *vs.* 0.01 M KCl the effect would be to increase the concentration of 0.001 M KCl relatively more than that of 0.01 M KCl and hence to lessen the observed concentration effect.

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[6] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 495. These values are for cells where the value of R_3 is reduced by broad contacts (as in the present case) or by frequent action currents.

The fact that the concentration effect of NaCl is so low must mean that the mobility of Cl^- in X is greater than zero.

[7] What is said of E_1 also applies to a certain extent to E_2.

Hence those measurements are chosen which run high for an entire lot of cells but the average of such a lot is taken rather than the highest individual value since the latter might be due to an E.M.F. in some layer other than X.

We may calculate the relative[8] mobilities by the usual equation (for 19°C.)

$$\text{P.D.} = 0.058 \frac{u - v}{u + v} \log \frac{a'_1}{a'_2}$$

where a'_1 and a'_2 are the mean ionic activities of KCl in l_o and l. Since these must be equal to the mean ionic activities[9] a_1 and a_2 in the external solutions we may put $a'_1 = a_1 = \gamma c_1$ and $a'_2 = a_2 = \gamma c_2$ (where c_1 and c_2 are the external concentrations and γ is the activity coefficient) so that we have (the observed value being 54.7 mv.)

$$0.0547 = 0.058 \frac{u - v}{u + v} \log \frac{(0.899)\,0.01}{(0.965)\,0.001}$$

This gives $u \div v = 73.24$, and putting for convenience[10] $v = 1$ we have for the mobility of K^+ in X, $u_K = 73.24$.

In the same manner we have[9] for NaCl (where the observed P.D. is 20.9 mv.)

$$0.0209 = 0.058 \frac{u - v}{u + v} \log \frac{0.903\,(0.01)}{0.966\,(0.001)}$$

from which u_{Na}, the mobility of Na^+ in X, is 2.18.

There is a great difference in the significance of these values. It is evident from Fig. 4 that a considerable error in measuring the P.D. of the concentration effect will not make much difference in u when the P.D. is small, but it makes a very great difference as the P.D. approaches 56.215 (the theoretical maximum for KCl, allowing for activities). Hence the value of u_{Na} is reliable but that of u_K is not. The latter, however, may be satisfactorily obtained from the chemical effect, as

[8] Similar calculations have been made for *Valonia*. *Cf.* Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 445.

[9] The activity coefficients are taken from Scatchard, G., *J. Am. Chem. Soc.*, 1925, **47**, 648.

[10] It may be noted that we should not expect to be able to measure diffusion potentials in a non-aqueous layer (such as X presumably is) where the concentration of ions is probably small, were it not for the fact that considerable difference exists between u and v.

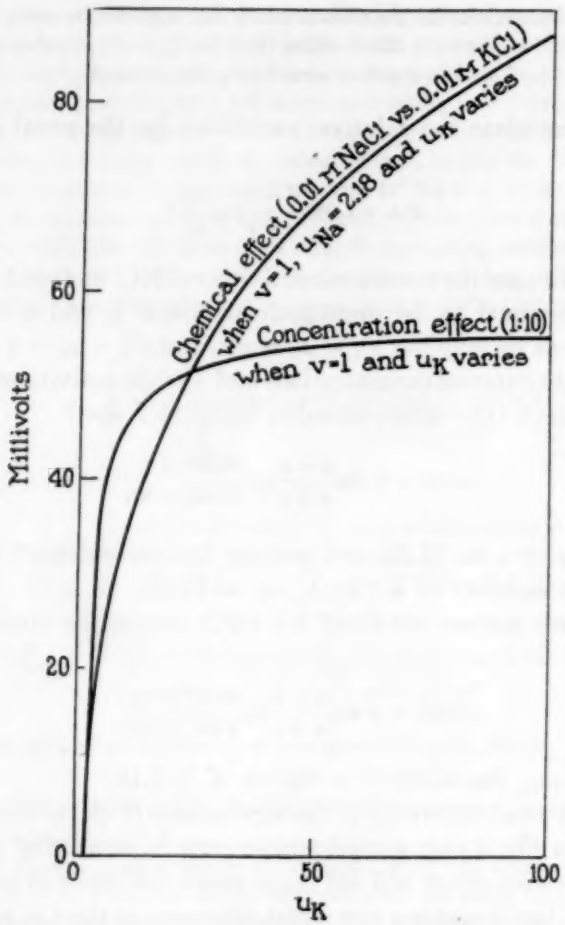

FIG. 4. Calculated concentration effect for tenfold dilution of KCl when $v = 1$ and u_K varies, and calculated chemical effect of 0.01 M NaCl against 0.01 M KCl when $v = 1$, $u_{Na} = 2.18$, and u_K varies.

is evident from Fig. 4 for the observed[11] P.D. of 0.01 M NaCl *vs.* 0.01 M KCl is 82.9 mv. and in this region the slope of the curve is such as to give reliable values.

We may employ the equation of Planck or that of Henderson, both

[11] The measurements were made as in determining the concentration effect of KCl, using 0.01 M NaCl in place of 0.001 M KCl, so that a_1 becomes the activity of 0.01 M NaCl.

of which in this case reduce to the same formula. If the concentration of KCl in the surface of X is equal to that of NaCl (and the temperature 19°C.) the formula is[12]

$$0.0829 = 0.058 \log \frac{u_K + v}{u_{Na} + v} = 0.058 \log \frac{u_K + 1}{2.18 + 1}$$

This gives $u_K = 85.45$.

If we calculate the P.D. of 0.01 M KCl *vs.* 0.001 M KCl by means of this value we have

$$\text{P.D.} = 0.058 \frac{u_K - v}{u_K + v} \log \frac{8.99}{0.965}$$

$$= 0.58 \frac{85.45 - 1}{85.45 + 1}$$

$$= 54.9$$

The observed value is 54.7.

This may be checked by measuring the P.D. of KCl 0.01 M against mixtures of Na and K. We may employ Henderson's formula,[13] which in this case reduces to

$$\text{P.D.} = 0.058 \log \frac{U_I + V}{U_{II} + V}$$

where $U_I = (u_K)$ (conc. K^+ in Sol. I) + (u_{Na}) (conc. Na^+ in Sol. I), $U_{II} = (u_{Na})$ (conc. Na^+ in Sol. II), and $V = (v_{Cl})$ (conc. Cl^-).[14]

When Solution I contains 0.005 M KCl + 0.005 M NaCl, and Solution II contains 0.01 M NaCl we have $U_I = (85.45)\ (0.005) + (2.18)\ (0.005)$, $U_{II} = (2.18)\ (0.01)$, and $V = (1)\ (0.01)$ so that we have

$$\text{P.D.} = 0.058 \log \frac{0.4273 + 0.01}{0.0218 + 0.01} = 66.0 \text{ mv.}$$

The observed value is 68.8 mv.

[12] For a brief discussion of Henderson's and Planck's formulae the reader may be referred to Michaelis, L., Hydrogen ion concentration, Baltimore, 1926.

[13] This involves the assumption that the concentration of K^+ in X bears the same relation to that of Na^+ as in the aqueous solution. There is no harm in making this tentatively. It also involves the assumption that the electrolyte concentration is the same throughout the boundary between NaCl in X and KCl in X, as is the case at the very start before diffusion has had time to accomplish much.

[14] The concentration of Cl^- is the same in both solutions.

It may be remarked in passing that there is a theoretical advantage in measuring the system $a'_2 \mid a'_1$ (successive solutions on the same spot) rather than $a'_2 \mid a'_3 \mid a'_1$ (different solutions on different spots) for it is well known from experiments *in vitro* that in general we should expect different values in the two cases (and this is in accord with Henderson's equation).[14a] This of course would not apply in the case of phase boundary potentials.

The equations of Planck and of Henderson take no account of activities but this is remedied by some of the more recent formulae. If, for example, we could assume that (as in the case of Henderson's equation) the electrolyte concentration is the same throughout the boundary between NaCl in X and KCl in X (as is the case at the very start before diffusion has had time to accomplish much) and that at each concentration ionic activities and conductances are approximately independent of the accompanying (monovalent) ions, the partition coefficient and dissociation in X being approximately the same for NaCl and KCl, we might adopt the equation used by MacInnes and Jones[15] for aqueous solutions and write

$$FdE = RT\,(T_{\mathrm{Na}} d \ln a'_{\mathrm{Na}} + T_{\mathrm{K}} d \ln a'_{\mathrm{K}} - T_{\mathrm{Cl}} d \ln a'_{\mathrm{Cl}})$$

in which T_{Na}, T_{K} and T_{Cl} are the transference numbers of the ions for mixtures in X. According to MacInnes this leads to the equation (at 19°C.)

$$E = 0.058 \log \frac{(1 - N_{\mathrm{K}}) - x_2(N_{\mathrm{Na}} - N_{\mathrm{K}})}{(1 - N_{\mathrm{K}}) - x_1(N_{\mathrm{Na}} - N_{\mathrm{K}})}$$

where N_{Na} and N_{K} are the transference numbers of the ions in solutions of the pure salts in X, and x_1 and x_2 are the molar proportions of NaCl in the external aqueous solutions (and by assumption in l_o and l).

[14a] In *Nitella* there is often no great difference between the two measurements.

[15] MacInnes, D. A., *J. Am. Chem. Soc.*, 1921, **43**, 1217. MacInnes, D. A., and Yeh, Y. L., *J. Am. Chem. Soc.*, 1921, **43**, 2563. MacInnes, D. A., and Jones, P. T., *J. Am. Chem. Soc.*, 1926, **48**, 2831. MacInnes, D. A., and Cowperthwaite, I. A., *Trans. Faraday Soc.*, 1927, **23**, 400. Smith, E. R., *Bureau of Standards J. Research*, 1929, **2**, 1137.

Some of the assumptions mentioned in the text may be unnecessary.

When NaCl is absent from one solution so that x_1 (as in the present case) becomes zero we have

$$E = 0.058 \log \frac{(1 - N_K) - x_2(N_{Na} - N_K)}{1 - N_K}$$

The transference number of Na^+ is $2.18 \div 2.18 + 1 = 0.6858$ and that of K^+ is $85.45 \div 85.45 + 1 = 0.98839$. Substituting these values we have for 0.01 M NaCl *vs.* 0.01 M KCl (where $x_2 = 1$)

$$\text{P.D.} = 0.058 \log \frac{(1 - 0.98839) - (0.6858 - 0.98839)\, x_2}{1 - 0.98839}$$

$$= 0.058 \log (1 + 26.063\, x_2)$$

$$= 83.08 \text{ mv.}$$

For 0.01 M NaCl *vs.* 0.005 M KCl + 0.005 M NaCl (where $x_2 = 0.5$) we have

$$\text{P.D.} = 0.058 \log (1 + (26.063)\,(0.5))$$

$$= 65.62 \text{ mv.}$$

Both of these are in close agreement with the other calculations.

These calculations may be checked by considering relative conductances which may be calculated by means of the formula

$$\text{Relative conductance}[16] = \frac{\alpha C'_{KCl}\,(u_K + v)}{\alpha C'_{NaCl}\,(u_{Na} + v)}$$

where α is the degree of dissociation and C' is the concentration in X. Assuming these to be approximately the same[17] for KCl and NaCl we have

$$\text{Relative conductance} = \frac{85.45 + 1}{2.18 + 1} = 27.19$$

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[16] As usually employed this is specific conductivity but for comparative purposes as in this case two conductances under the same conditions may be used.

[17] If the values of C'_K and C'_{Na} are equal it does not follow that the concentration in X of undissociated molecules (which may be the form in which penetration chiefly occurs) is equal since the dissociation constants may differ. For this reason and also because permeability may be determined by Y rather than X we need not look for a close correspondence between P.D. and permeability.

According to Blinks[6] the observed value[18] in the most reliable range (about 0.1 M) is from 25 to 50 (*i.e.*, the resistance per square centimeter in contact with 0.1 M KCl is 4000 ohms and in contact with 0.1 M NaCl is 100,000 to 200,000 ohms).

Calculation of Phase Boundary Potentials

Let us now consider calculations on the basis of phase boundary potentials alone. For the P.D. of 0.01 M NaCl *vs.* 0.01 M KCl we have

$$E = Pb_2 - Pb_1$$

where Pb_2 is the phase boundary potential of 0.01 M KCl at the outer surface of X and Pb_1 that of 0.01 M NaCl. If we employ the usual equations[19] (which take no account of activities and are of very doubtful validity) we may put (since the observed P.D. is 82.9 mv.)[20]

$$0.0829 = \frac{0.058}{2} \log \frac{A_K}{A_{Na}}$$

where A_K and A_{Na} are the "true" partition coefficients[19] of K^+ and Na^+ between the external solution and X. From this $A_K \div A_{Na} = 722.27$ and we may for convenience put $A_K = 722.27$ and $A_{Na} = 1$.

For 0.01 M NaCl *vs.* 0.005 M KCl + 0.005 M NaCl we should then have[20]

$$\text{P.D.} = 0.029 \log \frac{A_K C_K + A_{Na} C_{Na}}{A_{Na} C_{Na}}$$

$$= 0.029 \log \frac{(722.27)\,(0.005) + (1)\,(0.005)}{(1)\,(0.01)}$$

$$= 74.2 \text{ mv.}$$

The observed value is 68.8 mv.

The concentration effect presents greater difficulty. It is well known that no concentration effect is to be expected from phase

[18] It should be remembered that the measurements by Blinks have to do with the resistance across the whole protoplasm while the calculations of mobility may apply only to X.

[19] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, **11**, 83.

[20] In this calculation concentrations are employed instead of activities merely for convenience.

boundary potentials unless we make special assumptions. Were this done as suggested by Beutner,[21] for example, we should expect the same concentration effect for KCl as for NaCl. This is not true of *Nitella* where the concentration effect of NaCl is much smaller, as would be predicted on the basis of diffusion potentials since KCl is strongly negative to NaCl.

If, however, we assume that the "true" partition coefficients vary with concentration as well as with the nature of the ion we might avoid this difficulty, but we should be obliged to assume great variation. For example, if the "true" partition coefficient of K^+ at the concentration 0.1 M be called $A_{K\,0.1}$ and that at the concentration 0.001 M be called $A_{K\,0.001}$ and if the observed P.D. between 0.1 and 0.001 M KCl be[1] 116 mv. (A_{Cl} being constant) we have

$$\text{P.D.} = 0.116 = 0.029 \log \frac{A_{K\,0.1}}{A_{K\,0.001}}$$

whence[22] $A_{K\,0.1} \div A_{K\,0.001} = 10{,}000$.

To calculate the relative conductance we might assume as a first approximation that the degree of dissociation and the mobilities in X are about the same for KCl and NaCl and we should then have

$$\text{Relative conductance} = \frac{\alpha C'_K (u_K + v)}{\alpha C'_{Na} (u_{Na} + v)}$$

$$= \frac{C'_K}{C'_{Na}}$$

where C'_K is the concentration of K^+ and C'_{Na} that of Na^+ in X. Using the equations given above the value of $C'_K \div C'_{Na}$ may be obtained as follows: we have[19]

$$\text{P.D.} = 0.058 \log \sqrt{\frac{A_K}{A_{Na}}}$$

$$= 0.058 \log \frac{C_K A_K}{C'_K} \frac{C'_{Na}}{C_{Na} A_{Na}}$$

[21] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920. Michaelis, L., Hydrogen ion concentration, Baltimore, 1926. Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, **11**, 83.

[22] This value would, of course, be smaller if A_{Cl} varied in the opposite way from A_K.

where C_K is the concentration of K^+ in the external solution and C'_K its concentration in X, etc. Since $C_K = C_{Na}$ we have

$$\sqrt{\frac{A_K}{A_{Na}}} = \frac{A_K}{A_{Na}} \frac{C'_{Na}}{C'_K}$$

Dividing both sides by

$$\sqrt{\frac{A_K}{A_{Na}}} \frac{C'_{Na}}{C'_K}$$

we have

$$\frac{C'_K}{C'_{Na}} = \sqrt{\frac{A_K}{A_{Na}}}$$

In the present case we have

$$\text{Relative conductance} = \frac{C'_K}{C'_{Na}} = \sqrt{\frac{A_K}{A_{Na}}} = \sqrt{722.27} = 26.88$$

It is evident that we can approach the observed value of 25 to 50 by putting u_{Na} approximately equal to u_K, but this seems improbable in view of the fact that K and Na differ so widely in their behavior toward protoplasm.

Calculation of Mixed Potentials

It is clear from all that has preceded that the calculations are very satisfactory if we consider that phase boundary potentials play such a subordinate rôle that they may be neglected and this would be in harmony with recent theoretical tendencies.[23]

If, however, we grant that there may be cases where both phase boundary potentials and diffusion potentials play an important rôle it may be of interest to see what values are obtained by assigning equal weights to phase boundary potentials and diffusion potentials by putting $Pb_2 - Pb_1 = Pd_2$ in the equation $E_{conc.} = Pb_2 - Pb_1 + Pd_2$ (which gives the value of the concentration effect). For 0.01 M *vs.* 0.001 M KCl we should then have $0.0547 = Pb_2 - Pb_1 + Pd_2$ whence $Pd_2 = \frac{0.0547}{2} = 0.02735 = \frac{u_K - 1}{u_K + 1} (0.058) \log \frac{8.99}{0.965}$,

[23] Guggenheim, E. A., *J. Phys. Chem.*, 1929, **33**, 842. Cremer, M., *Handb. norm. u. path. Physiol.*, 1928, **8**, pt. 2, 1034 (especially the remark concerning Debye).

whence $u_K = 2.895$ and in similar fashion we obtain $u_{Na} = 1.456$. We may call the phase boundary potential, when the protoplasm is in contact with 0.01 M NaCl, Pb_3 and put

$$Pb_3 - Pb_3 = \frac{0.0829}{2} = 0.029 \log \frac{A_K}{A_{Na}}$$

whence $A_K \div A_{Na} = 26.87$ or putting $A_{Na} = 1$ we have $A_K = 26.87$.

Using these figures we may calculate the P.D. of 0.01 M NaCl *vs.* 0.005 M NaCl + 0.005 M KCl. We then have

$$\text{P.D.} = 0.058 \log \frac{U_I + V}{U_{II} + V} + 0.29 \log \frac{C_K A_K + C_{Na} A_{Na}}{C_{Na} A_{Na}}$$

$$= 0.00648 + 0.0419$$

$$= 48.4 \text{ mv.}$$

The observed value is 68.8 mv.

To calculate the relative conductance we should have (assuming the degree of dissociation of KCl in X to be about the same as that of NaCl)

$$\text{Relative conductance} = \frac{C'_K(u_K + v)}{C'_{Na}(u_{Na} + v)}$$

$$= \sqrt{26.87}\left(\frac{3.895}{2.456}\right)$$

$$= 8.22$$

Since the observed value is 25 to 50 it is evident this method of calculation is unsatisfactory.

The assumption that phase boundary potentials are as important as diffusion potentials in the concentration effect does not appear to be a fortunate one.

DISCUSSION

It is clear that for purposes of calculation it is much better to proceed as if diffusion potentials predominated. As the ionic mobilities found by these calculations are unlike those in water the nature of the protoplasmic surface becomes an important question. This surface is usually regarded as liquid since protoplasm in contact with water

(either occurring naturally or when squeezed out of the cell by pressure) commonly rounds up like an oily liquid, but this might occur if it were covered with a very thin solid film, comparable to a drop of mercury with a film of oxide on its surface. Plant protoplasm which secretes cellulose may possibly have on its outer surface a very thin film (solid or semisolid) of something like cellulose, which may adhere to the protoplasm and pull away from the cell wall during plasmolysis (but such a film seems much less probable in the case of the inner protoplasmic surface which surrounds the vacuole in most plant cells).

The mention of cellulose raises the question whether the cell surface acts like certain collodion membranes whose P.D.'s (somewhat resembling those of *Nitella*) are, according to Michaelis,[24] explainable on the basis of diffusion potentials. (Collander,[25] and Northrop[26] state that these membranes resemble the protoplasmic surface in some other respects.)

As nothing could be more welcome than a satisfactory model of the cell surface it may be desirable to inquire whether collodion really suffices for this purpose. Aside from the necessity of supposing the cell surface to be always solid there are difficulties. The concentration effects, for example, which are almost identical for HCl, KCl, NaCl, and LiCl with collodion, differ greatly[27] in *Nitella*. For collodion the ionic series is the same as with water (as far as investigated: Cs has not been studied); if this applied to *Nitella* each ion in the series Cs, Rb, K, Na, Li should be positive to all those preceding it and negative to all those following it. This is by no means the case: for example K and Rb are[28] strongly positive to Cs, and likewise tetraethyl ammonium fails to show the positivity to Na which is expected.

The fact that the ionic series is not the same as for water would be

[24] Michaelis, L., and others. Articles in *J. Gen. Physiol.*, 1925–29, **9–12**. Molecular physics in relation to biology, *Nat. Research Council Bulletin*, 1929, No. 69, 119.

[25] Collander, R., *Kolloidchem. Beih.*, 1924, **19**, 72; 1925, **20**, 273; *Soc. sci. Fennica, commentationes biol.*, (6) 1926, **2**, 1.

[26] Northrop, J. H., *J. Gen. Physiol.*, 1927–28, **11**, 233; 1928–29, **12**, 435; 1929–30, **13**, 21.

[27] This will be discussed in later papers.

[28] A similar effect is reported for muscle by Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926, 650.

intelligible if the outer protoplasmic surface were non-aqueous and we should expect the order to be an inverse function of the atomic weights unless disturbed by attractions between the ions and the non-aqueous phase producing results similar to hydration in aqueous media.

In this connection we need not detail all the features which must exist in a non-living model before it can be called completely satisfactory, but it may be desirable to emphasize certain fundamentals.

(1) The surface of a growing cell must admit both anions and cations or undissociated molecules of electrolytes. Höber[29] suggests that the surface of such cells is comparable to a mosaic of positive and negative collodion membranes: but how could such a mosaic be created? Is it not simpler to suppose that the protoplasmic surface is a non-aqueous phase through which salts pass by forming molecules, just as HCl passes through air from one aqueous solution to another by temporarily forming molecules? In this respect air may act in somewhat the same way as the protoplasmic surface but various non-aqueous substances, such as amyl alcohol, may furnish a closer analogy. Such formation of molecules would permit only a slow passage but certain substances with molecules preformed in the solution (*e.g.*, NH_3, H_2S, CO_2) show a more rapid passage through air, amyl alcohol, and protoplasm. But not all molecules pass out with equal facility; for example, we observe (with air, with amyl alcohol, and with protoplasm) a ready passage of alcohol but not of sugar.

The chemical and concentration effects indicate that certain ions enter X and it is of course possible that ion pairs, such as $Na^+ + Cl^-$, penetrate rapidly enough to play an important rôle in permeability or that H^+ and organic anions, produced in the cell, are exchanged for Na^+ and Cl^-, but the high electrical resistance of the protoplasm for most ions, observed by Blinks,[6,30] does not favor this idea. If the outer surface of the protoplasm is non-aqueous (lipoid, for example) it would presumably contain a higher concentration of molecules than of ions so that (unless the mobility of ions greatly exceeds that of molecules) penetration would be largely in molecular form.

(2) Subjecting normal cells of *Nitella* or *Valonia* to an electric

[29] Höber, R., and Hoffmann, F., *Arch. ges Physiol.*, 1928, **220**, 558.
[30] Blinks, L. R., unpublished results.

current produces a series of effects (*e.g.*, back E.M.F. with direct current, falling off of impedance as a function of the frequency with alternating current) not imitated by collodion or by amyl alcohol or other non-living models except perhaps by metallic electrodes.[6]

(3) In many cases the protoplasmic surface undergoes great and rapid changes in permeability with complete or partial recovery which have not yet been satisfactorily imitated by any model.

In view of these and other complications the question of a model appears to need further investigation.

SUMMARY

The P.D. of 0.01 M *vs.* 0.001 M KCl (concentration effect) in *Nitella* is 54.7 mv. and that of NaCl is 20.9 mv. The P.D. of 0.01 M NaCl *vs.* 0.01 M KCl is 82.9 mv.

If we assume that diffusion potentials predominate we may calculate the relative mobilities of the ions in the outer layer of the protoplasm. If we put $v_{Cl} = 1$ we obtain $u_K = 85.45$ and $u_{Na} = 2.18$. (These values depend upon the fact that the mean ionic activities in this layer must be equal at equilibrium to the mean ionic activities in the external solution.)

Using these values the calculated P.D. of 0.01 M NaCl *vs.* 0.005 M NaCl + 0.005 M KCl is 66 mv. (the observed value is 68.8 mv.) and the conductance of KCl in the outer layer of the protoplasm is 27 times as great as that of NaCl (the observed conductance for the whole protoplasm is 25 to 50 times greater for KCl than for NaCl).

Calculations on the basis of phase boundary potentials are less satisfactory.

THE CELL SAP OF HALICYSTIS

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I

Knowledge of the chemical constitution of the vacuolar sap of *Halicystis*, found in Bermuda, has been based on the analyses by Dorcas,[1] which were performed with sap extracted from cells floated in upon the beaches.[2] During the past 5 years, samples of sap have several times been extracted from smaller cells of *Halicystis* at Bermuda, detached by hand from the substratum upon which they were growing. The quantity of this sap was not sufficient for complete analyses, but the ratio of potassium to sodium was in every case found to be very low, and in good agreement with that reported in the original analysis. This resemblance has been noted in a paper[3] dealing with the potential differences of *Halicystis* cells collected from their position of growth in 1929.

A more complete analysis is now presented, based on an adequate sample of sap from growing cells.

The material was collected in April, 1930, the temperature of the water being 19°C. The cells grow on crusts of *Lithothamnion* just below low tide, often occurring in colonies up to several hundred, and range from less than 1 mm. to more than 12 mm. in diameter. The sap was extracted from those between 3 and 7 mm. in diameter. They were separated from the colonies within 1 day after collection, by breaking up the *Lithothamnion* crust into bits which bore one or more cells (each attached by the characteristic solitary pedicel). These were drained

[1] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, **7**, 633.

[2] These were erroneously called "*Valonia ventricosa*." For the analysis of sap from correctly identified *V. ventricosa* see Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, **68**, 164.

[3] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 223.

on a towel, and carefully dried with filter paper before the extraction of sap. The tendency of the cells to collapse when punctured (which has been noted before)[3] is such that much of the sap is lost by spurting if the attempt is made to collect it in a capillary pipette. Instead of this, the cells were inverted and punctured at the rounded apex by a glass needle, along which the sap flowed to a bend, where it dropped off into a Pyrex vessel.

Only the sap which flowed out freely was used, and to prevent the expulsion of protoplasm complete collapse was avoided. Most of the cells recovered their original volume and turgor within 2 or 3 days after their return to sea water, showing that the protoplasm had not been seriously damaged.

Over 300 cells from three different collections were punctured, to obtain 25 cc. of sap, which was clear and practically free of chloroplasts. Its specific gravity at 21°C. was 1.0258 as compared with 1.0277 for sea water. These values agree very well with those which Dorcas obtained with sap from the stranded cells. They indicate why the larger cells are able to float when detached from the substrate. (Smaller cells may be held down by the weight of wall, protoplasm, and pedicel; only attached, non-floating cells were used for the extraction of the sap here analyzed. All cells, however, stand upright in sea water, and tug at their attachment like captive balloons.)

II

The details of analysis follow.

a) Sodium and Potassium.—A 10-cc. sample was transferred from the total volume of sap to a 25 cc. volumetric flask, and treated first with 10 cc. of absolute alcohol plus ammonium oxalate in the approximate ratio of 9:1. Then 5 cc. of the Schaffgottsche[4] ammonium carbonate reagent was added and the total volume made up to exactly 25 cc. by adding a drop or two of water (to compensate the contraction due to dilution of the alcohol). The flask was then tightly stoppered to prevent loss of alcohol by evaporation, and set aside long enough to permit all the calcium and magnesium to be precipitated as oxalate or carbonate. The precipitate was removed by centrifugalizing at about 2500 revolutions per minute in covered tubes. Two 10-cc. samples, each equivalent to 4 cc. of original sap, were then withdrawn and the sodium and potassium determined.

After evaporation and gentle ignition in quartz the total weight of salt present was determined. This was assumed to consist of potassium and sodium chlorides exclusively, since only negligible amounts of other anions were found. A test with barium chloride on an acidified 0.5-cc. sample of the sap showed the presence

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[4] Treadwell, F. P., and Hall, W. T., Analytical chemistry, 1924, 6th ed., **2**, p. 79.

of an unweighable trace of sulfate. Carbonate ion, which might have been present in the original sap, could not have survived the ignition in the ammoniacal solution. Bromide and iodide were found to be absent by treating portions of the sap with chlorine water and extracting with chloroform. Nitrate was found to be absent by the brown ring test.

Potassium was determined as potassium perchlorate. Owing to the small amount present, the weight of the salt was only 0.0035 gm. For this reason the error in this determination may be rather large, ±3 per cent. Since the sodium was determined by difference the value assigned to it is subject to an error due to the error of the potassium in addition to the ordinary error of weighing. However, the amount of sodium is so much greater than the amount of potassium that this extra error is not significant. The sodium value is correct to about ±0.4 per cent.

b) Calcium and Magnesium.—These were determined in a single separate 10-cc. portion of the original sap. The calcium was precipitated as oxalate according to the procedure of Treadwell[5] and weighed as calcium oxide after ignition in platinum. The estimated error is ±1 per cent.

The magnesium was precipitated as magnesium ammonium phosphate from the filtrate of the calcium determination. It was then determined as magnesium pyrophosphate by the method of B. Schmitz.[6] The estimated error is ±0.5 per cent.

c) Halide.—The halide was determined in two samples of 1 cc. each by Mohr titration with silver nitrate. The error of this determination is about ±0.5 per cent.

d) Ammonia and Hydrogen Ions.—Since the samples for the above determinations had been kept in the ice box for 10 days after extraction, a fresh sample of about 4 cc. was extracted for the determination of ammonia, and hydrogen ion concentration. Ammonia was determined in two samples of 1 cc. each, by treating with sodium hydroxide and distilling over into standard acid. One of the standard acid samples was titrated with a standard alkali, and the second was Nesslerized. The amount of acid neutralized by the ammonia was no greater than the possible error in reading the burette. The Nessler test showed a very faint trace of ammonia.

The pH of the fresh sap was found to be 6.2, by use of chlor-phenol red (allowing for salt error).

The transfer pipettes used in this work were all certified by the Bureau of Standards to be correct within the permitted tolerance. The burette used for halide had received a certificate from its manufacturer, showing that it had only negligible corrections. All other volumetric apparatus had been calibrated in this laboratory.

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[5] Treadwell, F. P., and Hall, W. T., *loc. cit.*, p. 86.

[6] Treadwell, F. P., and Hall, W. T., *loc. cit.*, p. 78.

III

The analysis follows:

Chloride	0.6028 moles per liter
Sodium	0.5570 " " "
Potassium	0.0064 " " "
Calcium	0.0080 " " "
Magnesium	0.0167 " " "
Sulfate	trace
Total cations	0.5881 " " "
	0.603 gm. equivalents per liter

The molar ratio of potassium to sodium is 0.0114. This is even lower than the ratio found in the stranded cells,[1] which was 0.0278. The exact ratio is apparently variable since determinations made in 1929 gave 0.0314 for stranded cells, and 0.0052 for growing cells. An earlier ratio found by Dorcas in a small sample of sap from growing cells was 0.04. In most of these cases the K:Na ratio differs from that in the sea water, and is as often reduced as it is increased. The best values for the growing cells show a partial exclusion of potassium, its concentration being 0.0029 molar and 0.0064 molar, while the sea water has about 0.0125 molar. Thus the sap has only 0.23 to 0.51 as much potassium as the sea water. The stranded cells accumulate potassium slightly, having 1.3 to 1.4 times as much as the sea water. The new analyses emphasize more than ever the striking deficiency of potassium in the sap of the Bermuda *Halicystis*. In every case its concentration is less than 4 per cent of that in the sap of *Valonia macrophysa*, in which the lowest K:Na ratio found at Bermuda is 2.55 (unpublished data). The ratio runs up to 5.72 in this species, and to 18.5 in *V. ventricosa*.[2] Large differences between the saps of *Chara ceratophylla* and *Nitella clavata* have recently been demonstrated by Collander's detailed analysis[7] of the former, and *Nitella* sap has also been shown to vary considerably in its K:Na ratio.[1,8] In view of these variations it is perhaps not surprising that the sap of the Pacific Coast *Halicystis ovalis* is very different from that of the Bermuda form; its K:Na ratio of 1.5 approaches that of *Valonia macrophysa*.[9]

[7] Collander, R., *Acta Botanica Fennica*, 1930, **6**, 1.

[8] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, **5**, 629.

[9] Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 409.

In the present state of our knowledge these differences can only be called "specific." The Bermuda form differs markedly from the *H. ovalis* of Europe in morphological details, and reaches a greater size. It is being proposed elsewhere as a new species. In the absence of sap analyses for *H. ovalis* and *H. parvula* of Europe it has a unique position on account of its extreme divergence from the *Valonia* types. Its value in comparative studies is thereby enhanced.

SUMMARY

Analysis was made of 25 cc. of vacuolar sap, obtained from over 300 cells of *Halicystis* collected in their position of growth in Bermuda. The determinations agree very well with those of the stranded cells, except that there is even less potassium present. The K:Na ratio is 0.0114.

CRYSTALLINE PEPSIN

I. Isolation and Tests of Purity

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I

INTRODUCTION

Enzymes are in many respects connecting links between living and inanimate matter since their action is analogous to inorganic catalysts, although the enzymes themselves are found only in living organisms. As catalysts they increase the rate of one or more specific reactions and so act as directive agents for the reactions occurring in the organism. This directive property is undoubtedly essential for the existence of living cells. As a consequence of these properties the study of enzymes has been of interest to both chemists and biologists and has resulted in a great increase in the knowledge of their mode of action. The results of attempts to isolate the enzymes in pure form, however, have been singularly unsuccessful. There seems to be no convincing evidence that any enzyme has been obtained in the pure state; and only one, the urease described by Sumner (1), has been previously obtained in crystalline form. A number of methods have been found which allow the activity of an enzyme preparation to be increased almost indefinitely; at the same time, however, the preparation becomes more unstable and eventually the activity becomes lost.

In practically all the work the assumption has been made either explicitly or otherwise that the activity was a measure of the purity of the preparation and that any increase in activity was due to an increase in purity. This is not necessarily true. If the enzymes are analogous to inorganic catalysts then it is quite possible that the activity depends on the physical arrangement of the molecules or atoms (2). Evidence for this relation between the physical state and

the activity was found by Fodor (3) in the case of the proteolytic enzymes of yeast, and by Kuhn and Wasserman (4) in the case of hemin. It is possible, on the other hand, that enzymes in general are of the type of hemoglobin (which might be considered an enzyme), and that they consist of an active group combined with an inert group. It might be possible under certain conditions to attach many more active groups to the inert group and so increase the activity above that of the original compound. Either of the above ideas would account for the well-known fact that crude preparations are much more stable than purified material and that the rate of inactivation of enzyme solutions practically always shows evidence of a mixture of stable and unstable forms (5, 6).

There is some reason to think, therefore, that enzymes exist in a more stable form for either physical or chemical reasons, and in view of the uniformly negative results which have been obtained in attempting to isolate the most active preparations it seemed advisable in attempting the isolation of pepsin to study the more stable as well as the most active fraction.

II

PRELIMINARY EXPERIMENTS

A number of methods have been proposed for the purification of pepsin, such as precipitation with safranin (7), etc., fractionation by various adsorbents, and precipitation by dialysis from acid solution (Pekelharing (8)). These and a number of other methods were tried and more or less active preparations obtained. The results with Pekelharing's method seemed the most encouraging, however, since the loss of activity was less and there was some indication that a constant activity was reached. This result has been reported by Pekelharing and also by Fenger, Andrew and Ralston (9) using a similar method. It was found, however, that the dialysis could be dispensed with and the process made more rapid and efficient by solution with alkali and subsequent precipitation with acid, after a preliminary precipitation with half saturated $MgSO_4$ or $(NH_4)_2SO_4$. The amorphous material so obtained contains about half the activity present in the original material and is 3 to 6 times as active as measured by the liquefaction of gelatin and about 5 times as active as measured by the

digestion of casein or by the rennet action on milk.[1] Repetition of this procedure gave products of increasing activity as measured by the liquefaction of gelatin, and apparently this activity could be increased indefinitely. Several samples were obtained which were 100 times as active as the original preparation. They were also more unstable, so that each succeeding precipitation was accompanied by a larger and larger percentage loss until finally no more active material remained. This has been the fate of all previous attempts to isolate the most active fraction of a number of enzymes. When the activity of the various fractions was determined by the rate of hydrolysis of casein or by the rennet action on milk, however, it was found that the activity increased until it reached about 5 times that of the crude preparation

[1] *Determination of Activity.*—The rate of digestion of proteins by pepsin may be followed by determining the increase in carboxyl groups or amino nitrogen, or by the decrease in protein nitrogen or by the changes in viscosity. The increase in carboxyl groups probably represents the best measure of the progress of the reaction and has been used as the basis of the activity units used in this paper.

The most widely used unit of enzyme activity in general is that suggested by Euler (16) and is equal to the velocity constant of the reaction divided by the grams of enzyme. Theoretically this is undoubtedly correct since the activity of a catalyst can be expressed only as a velocity constant. Many enzymes do not give a velocity constant independent of the substrate concentration but instead the velocity constant decreases as the substrate concentration increases. This is especially true of pepsin and in addition the reaction does not follow any simple reaction rate. The end point of the reaction, which must be known in order to calculate the velocity constant, is also very difficult to determine. It is not practical therefore to use the velocity constant as a measure of activity of pepsin.

The activity of pepsin may conveniently be defined as the milliequivalents of carboxyl groups liberated per mole or gram of enzyme per minute at 35.5°C., optimum pH, and 5 per cent substrate concentration. Theoretically it would be better to use that substrate concentration at which the rate of reaction is a maximum, but experimentally this is difficult, owing to the high viscosity of such concentrated solutions. The enzyme concentration used should be in the range in which the activity is proportional to the enzyme concentration. In order to determine the activity in this way the rate of hydrolysis of casein, gelatin, edestin, and denatured egg albumin at pH 2.0 to 2.5, was determined by means of the increase in formol titration (19).

The following abbreviations are used in this paper:

PU = proteolytic units = milliequivalents carboxyl groups per minute.
$[PU]_{gm.}$ = " " per gram.
$[PU]^{cas.F}_{gm.}$ = " " " " as determined by cas. F. method, etc.

and then remained constant instead of increasing as did the gelatin liquefying power. This was the result reported by Pekelharing and also by Fenger, Andrew and Ralston. This material appeared to be protein, as previous workers had found, and was reasonably stable. Efforts were therefore made to isolate this protein in crystalline form.

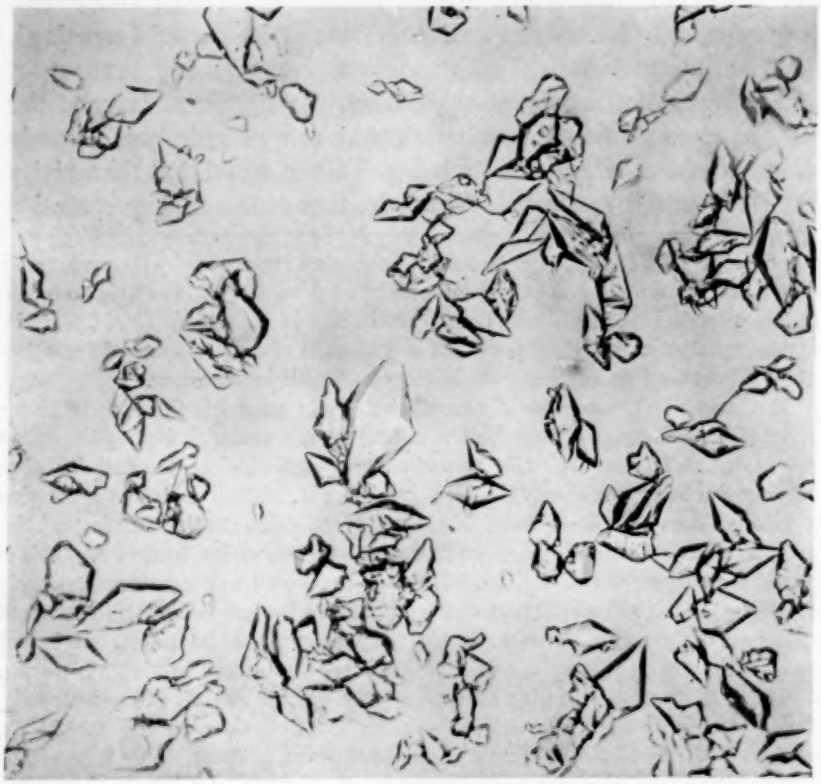

FIG. 1. Crystalline pepsin

III

Isolation of the Crystalline Enzyme

It was noticed that the precipitate which formed in the dialyzing sac when the procedure of Pekelharing was followed appeared in more or less granular form and filtered rather easily, as though it were on the verge of crystallization. This precipitate dissolved on warming the

suspension and it was eventually found that it could be induced to crystallize by warming to 45°C., filtering, and allowing the filtrate to cool slowly. The crystals so obtained were regular hexahedra and showed a tendency to grow in clusters, especially when appearing from more acid solutions. They are remarkably similar to the urease crystals pictured by Sumner and differ only in that they have a hexagonal base while the urease has an octagonal base. On one occasion a few crystals with truncated pyramids were obtained. They had the same activity and optical activity as the usual form. The crystals showed positive double refraction and were optically active in solution. They possessed proteolytic activity, when dissolved, equivalent to 5 times that of the U. S. P. 1 to 10,000 pepsin as measured by hydrolysis of casein, and 2.5 times as measured by the liquefaction of gelatin.

Improved Method for the Preparation of the Crystals.—The isolation of the crystals in bulk by the above method was difficult owing to the dialysis. It was found that this could be avoided and the purification carried out as outlined above for the amorphous preparations except that the acid precipitate was dissolved at 45° C. in concentrated solution. On inoculation, this solution set to a solid paste of crystals. The following is an outline of the method as finally developed.

IV

General Properties and Analysis of the Crystals

The material prepared in this way has the general properties of a protein. It is coagulated by heat, precipitated by saturation of the solution with $MgSO_4$ or $(NH_4)_2SO_4$ and gives a strongly positive xanthoproteic test. The Millon test is negative. The crude material contains a large amount of yellowish pigment which is removed with difficulty. It may be largely removed by reprecipitation with $MgSO_4$ and becomes less as the material is recrystallized. All the preparations however, give a slightly yellowish solution when dissolved although the dry crystals are pure white after several crystallizations. There is no relation between the activity and the color. Analysis of the material gave the results shown in Table II.

The crystals are difficult to dissolve after drying and are best kept under saturated $MgSO_4$ at 5°C. They are instantly inactivated by alkali in solution and lose activity slowly in acid solutions. The

TABLE I

Preparation of Crystalline Pepsin

Procedure	Activity per gm. dry weight Method			
	Gel. V.	Cas. S.	Rennet	E. A.
500 gm. Parke, Davis pepsin U. S. P. 1:10,000 dissolved in 500 ml. H_2O and 500 ml. 1 Normal H_2SO_4 added. 1,000 ml. saturated $MgSO_4$ added with stirring. Solution filtered through fluted paper (S. and S. No. 1450½) and then with suction. Filtrate discarded.	0.075	2.5	2.5	4.5
Precipitate 1. Wash twice with equal volume ¾ saturated $MgSO_4$, filter with suction. Filtrate discarded.				
Precipitate 2. Stir with water to thick paste and M/2 NaOH run in until complete solution. (Great care must be taken to avoid local excess of NaOH. pH never more than 5.0.) M/2 H_2SO_4 added with stirring until heavy precipitate forms, (pH about 3.0), 3 to 6 hrs. at 8°C., filter with suction. Filtrate discarded.	.15	7.5	7.5	7.0
Precipitate 3. Stir with H_2O to thick paste at 45°C., M/2 NaOH added carefully until precipitate dissolves, (filter if cloudy and discard precipitate). Beaker containing filtrate placed in a vessel containing about 4 liters of H_2O at 45°C., inoculated and allowed to cool slowly, cooling should require 3 to 4 hrs. and heavy crystalline precipitate should form at about 30 to 35°C. Solution kept at 20°C. for 24 hours. Thick crystalline paste, filter with suction.	.15	10.0	10.0	8.0
Precipitate 4. Wash with small amount of cold H_2O and then with ½ saturated $MgSO_4$ and store under saturated $MgSO_4$ at 5°C.	.17	14.0	[14.0]	9.0
Filtrate. M/2 H_2SO_4 added to pH 3.0, amorphous precipitate filtered off and treat as Precipitate 3.				

Recrystallization

Method 1. Crystalline paste filtered with suction on large funnel so as to form a thin layer of crystals and washed 3 times with cold M/500 HCl. Filter cake stirred to a paste with ½ its weight of water, the suspension warmed to 45°C. and M/2 NaOH run in slowly with constant stirring until the precipitate dissolves (pH<5.0). M/2 H_2SO_4 is then run in until the solution is faintly turbid, a few crystals added and the solution allowed to cool slowly as before. A heavy crop of crystals should separate in about 24 hrs. The suspension is then warmed to 45°C. again and more H_2SO_4 added until the pH of the suspension is about 3.0. It is then allowed to cool slowly again, and filtered after 24 hrs. The crystals may be washed with M/500 HCl until free of SO_4.

Ammonium sulfate may be used in place of $MgSO_4$. Sodium acetate may be used in place of sodium hydroxide.

Method 2. Crystals dissolved with NaOH and treated as described for Precipitate 2.

inactivated material is digested by the remaining active material and a large amount of tyrosin crystallizes out. This process also occurs slowly in the ice box so that the crystals on standing become mixed with nonprotein material that is not precipitated by salt nor by heat. The crystals can be freed from this soluble material by thorough washing with H_2O or by recrystallization. When freshly prepared in this way 98 to 99 per cent of the nitrogen is precipitated from solution by heating rapidly to boiling at pH 3 with sulfuric acid and Na_2SO_4, by saturation with $MgSO_4$ or $(NH_4)_2SO_4$, by the addition of alkali

TABLE II

Analysis. Dried at 60° in vacuo for 24 Hrs.

Method	Dumas	Kjeldahl	Van Slyke							Precipitation with $BaCl_2$
	Total N	Total N	Amino N	C	H	Cl	S	P	Ash	SO_4
Per cent dry weight	15.5	15.15	0.80	52.3	6.66	0.23	0.88	0.078	0.40	0
	15.3	15.30		52.6	6.64	.20	.82		.55	
					6.70					

TABLE III

Activity and Composition of Various Preparations of Pepsin, Crystallized Once

Preparation	1	2	3–5	5–10	10–12	12–20	22
$[PU]^{gel.\ V.}_{gm.}$	0.17	0.16	0.18	0.154	0.16	0.14	0.17
Per cent N	15.2	15.1	15.1	15.3	15.2	15.0	15.1
$[\alpha]_D^{22°}$ pH 4.5	−70	−72	−74	−73	−74	−71	−70

and subsequent neutralization, or by heating with 10 per cent trichloracetic acid.

Constant Activity of Various Preparations.—About 2 kg. of the crystals have been prepared from six different lots of the commercial preparation during the course of this work and have all had the same percentage of nitrogen and the same activity within the experimental error. A summary of these properties of the various preparations is given in Table III. The preparations in some cases were combined at the final crystallization.

The relative activity with various substrates and various methods is shown in Table IV. The figures are the average of 6 to 10 determinations with different enzyme preparations. All crystalline preparations tested yielded the same result with these methods, but amorphous preparations frequently gave much higher activity as measured by gelatin hydrolysis and lower activity as measured by egg albumin,

TABLE IV

Activity of Various Enzyme Preparations

Enzyme	Substrate	Method	Activity. Milliequivalents per gm. per min. = $[PU]_{gm.}$	Milliequivalents per millimole or equivalents per mole per min. = $[PU]_{mole}$
Crystalline pepsin	Casein	Formol	14 ± 0.5	500
		Solution	[14]	
		Rennet action	[14]	
	Gelatin	Formol	.17 ± 1	6
		Viscosity	[.17]	
	Edestin	Formol	28 ± .8	1,000
	Egg albumin	Formol	9 ± .5	320
U.S.P. pepsin 1:10,000	Casein	Formol	2.5	
		Solution	[2.5]	
		Rennet action	[2.5]	
	Gelatin	Formol	.075	
		Viscosity	[.075]	
	Egg albumin	Formol	4.5	

while the activity as measured by casein hydrolysis remained constant. On recrystallization of these abnormal amorphous precipitates the activity always returned to the characteristic value for the crystalline material, shown in the table.

The activity compared to other enzymes is less than that reported by Sumner for urease, on a weight basis, and much less than for several other enzymes, but comparison should be made on the basis of activity per mole rather than per gram of enzyme.

V

Evidence Concerning the Purity of the Crystalline Material

Constant Activity and Composition on Repeated Crystallization.— The work reported above shows that a crystalline protein having proteolytic activity may be obtained in quantity from commercial preparations of pepsin. The question of interest is whether this proteolytic activity is a property of the protein molecule or of another molecular species associated with the protein. The usual criterion for the purity of a substance is constant composition and properties after repeated recrystallization. Unfortunately in the case of proteins the

TABLE V

Summary of Recrystallized Pepsin

Crystallization No. and color	Quantity of crystals	$[PU]^{gel.\ V.}_{gm.}$	$[PU]^{cas.\ S.}_{gm.}$	N	P	$[\alpha]^{22°}_{D}$ pH 5.0	Formol per gm.
	gm.			*per cent*	*per cent*		*ml. 0.1 NaOH*
1. Dark brown.....	50	0.18	14.0	14.85	0.078	−70	14.0
2. Brown.....	22	.17	14.5	15.0	.080	−70	12.5
3. Yellowish.....	15	.15	15.0	15.14	.075	−68	12.5
4. Slightly yellow fine powder.....	9	.16	14.5	15.16	.076	−67.6	11.0
5. Nearly white.....	5	.16	14.8	15.13	.077	−68	11.0
6. " "	3	.14	14.5		.073	−72	11.0
7. " "	.5	.17	15.0	15.15	.080	−72	12.0
Original preparation....		.075	2.5				

melting point, which is the most sensitive test of purity, cannot be used since proteins decompose before melting. Of the other properties the proteolytic activity, optical rotation, and percentage of nitrogen and of phosphorus were considered the most significant and these properties were determined on preparations obtained from a series of seven successive crystallizations. A summary of the results of this experiment is shown in Table V. There is no perceptible drift in any of the properties determined and therefore no indication that the material can ever be separated into fractions by crystallization under these conditions. This shows that the composition of the crystals is independent of the concentration and quantity of the solution from which they are formed.

Several systems could give this result (10). (1) A mixture of two or more substances present in amounts which are proportional to their solubilities. (2) A solid solution of such composition as to have a minimum solubility. This would correspond to a constant boiling point mixture of miscible liquids. (3) A solid solution of two or more substances having nearly the same solubility. (4) A pure substance.

The various possibilities noted above all depend on some definite relation between the composition of the mixture and the solubilities of the hypothetical components. If this relation were changed, the composition would be changed (except in the case of allotropic modifications). The possibility of such a relation would be greatly reduced if the recrystallization were carried out in a number of different solvents in which the solubility was different. It is quite possible that a mixture of constant composition might be obtained from one solvent just as constant boiling mixtures are frequently found, but it would be expected that the composition of the mixture would vary with different solvents just as the composition of constant boiling mixtures varies with the pressure. If it could be shown therefore that the material retained its constant proteolytic power and composition after recrystallization from a number of different solvents, it would be excellent proof that it was a pure substance. Unfortunately, however, it is not possible to crystallize the material from a series of solvents, but the same result may be obtained by studying the solubility in several solvents.

Results of the Solubility Determinations.—The values for the solubility obtained with different amounts of precipitate in various solvents are shown graphically[2] in Figs. 2 to 6. Since the solubility is found to be independent of the amount of precipitate this procedure does not affect the shape of the curve. The soluble nitrogen per milliliter of solution, the proteolytic activity per milliliter of solution as determined by the digestion of casein or gelatin ($[PU]_{ml.}$) and the optical rotation of the solution in a 1 dm. tube are plotted against the total nitrogen content per milliliter of the suspension. The values corresponding to solutions obtained from the undersaturated side are represented by solid dots and those corresponding to solutions obtained from the supersaturated side

[2] In order to save space the scale representing the total amount of nitrogen per milliliter of suspension has been changed to a logarithmic one in some of the curves after the amount has become large as compared to the amount in solution.

are represented by circles. The solid lines drawn for the curves representing the pepsin per milliliter and the optical activity were calculated on the assumption that the ratio of activity or optical activity to nitrogen was the same in the solution as in the original crystals. That

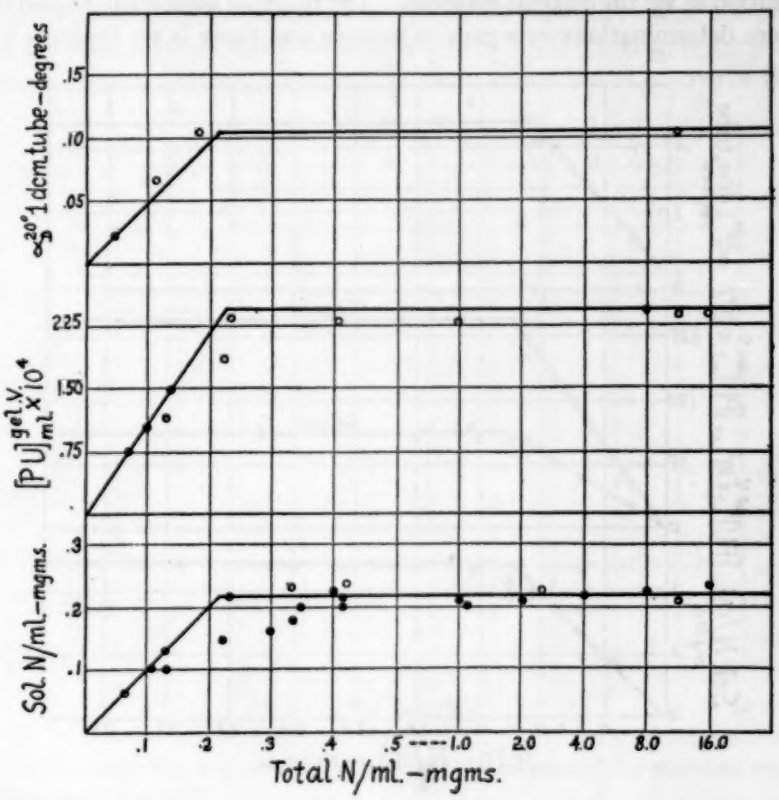

FIG. 2. Solubility of crystalline pepsin in 0.05 acetate and 0.5 saturated magnesium sulfate at 8°C.

is, 1.15 proteolytic units per gram of nitrogen as measured by the liquefaction of gelatin, 92 proteolytic units per gram of nitrogen as measured by the digestion of casein and a specific rotation per gram of nitrogen of $-460°$ as measured at 22 to 24°C. with the sodium D line.

The results show: (1) that the solubility is independent of the quan-

tity of solid present, (2) that the value for the solubility is an equilibrium value since it is the same whether obtained from supersaturated or undersaturated solutions, and (3) the specific proteolytic activity and the specific optical activity are the same for the material present in the solution as for the original material. The material behaves in regard to these determinations as a pure substance and there is no evidence of

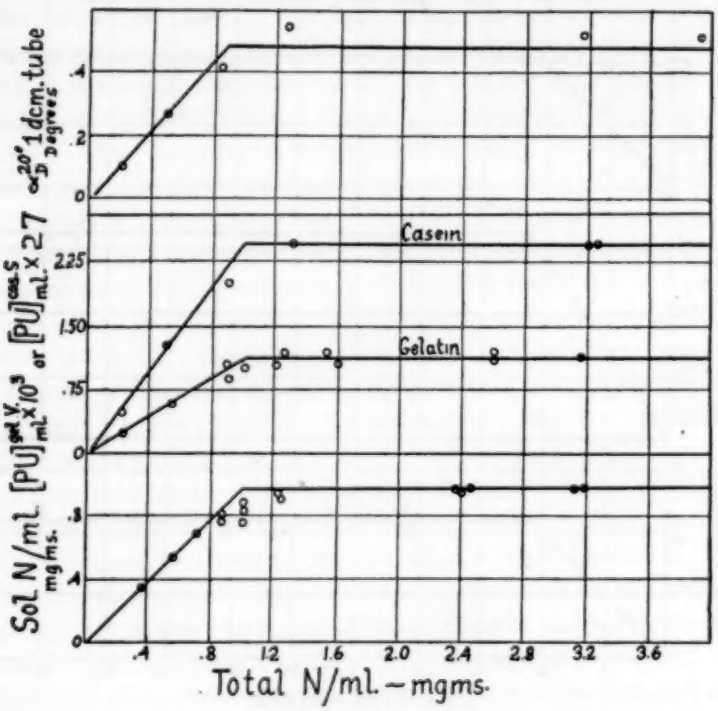

FIG. 3. Solubility of amorphous pepsin in 0.05 acetate and 0.5 saturated magnesium sulfate at 20°C.

mixture. They show conclusively that the activity cannot be ascribed to the presence of a minute amount of a very highly active material associated with the protein, unless it be further assumed either that this active material has about the same solubility as the protein or that it forms solid solutions with the protein. If the active material were soluble and *mixed* with the protein the activity per milliliter of solution

would continue to increase as more and more solid material was added. While if it were present as a relatively insoluble material the activity per milliliter of the supernatant solution would become constant before the solubility of the protein had become constant. The figures show, however, that the proteolytic activity of the liquid becomes constant at the same point at which the concentration of the protein in the solution becomes constant. The only mixture which would behave in this

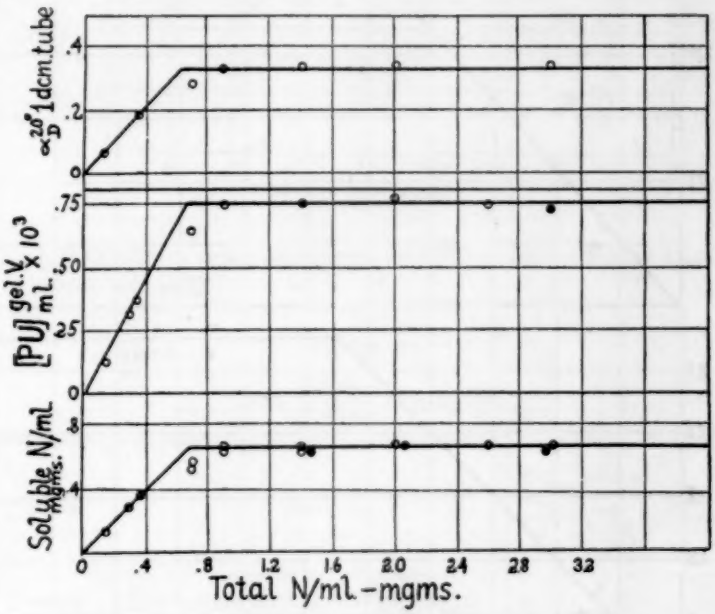

FIG. 4. Solubility of amorphous pepsin in 0.01 acetate and 0.5 saturated magnesium sulfate at 20°C.

way would be one in which the relative amounts of active material and protein were in almost exact proportion to the relative solubilities of the two substances. This might conceivably be the case in one solvent but the possibility that this relation would hold in several solvents appears so small as to be negligible.

There remains only the possibility that the active material forms a solid solution with the protein and is either present in small amount or has about the same solubility as the protein. If it were present in

small amount and dissolved in the protein, its presence would not affect the total solubility curve. The ratio of the active material to the protein in the precipitate at the point where the precipitate first

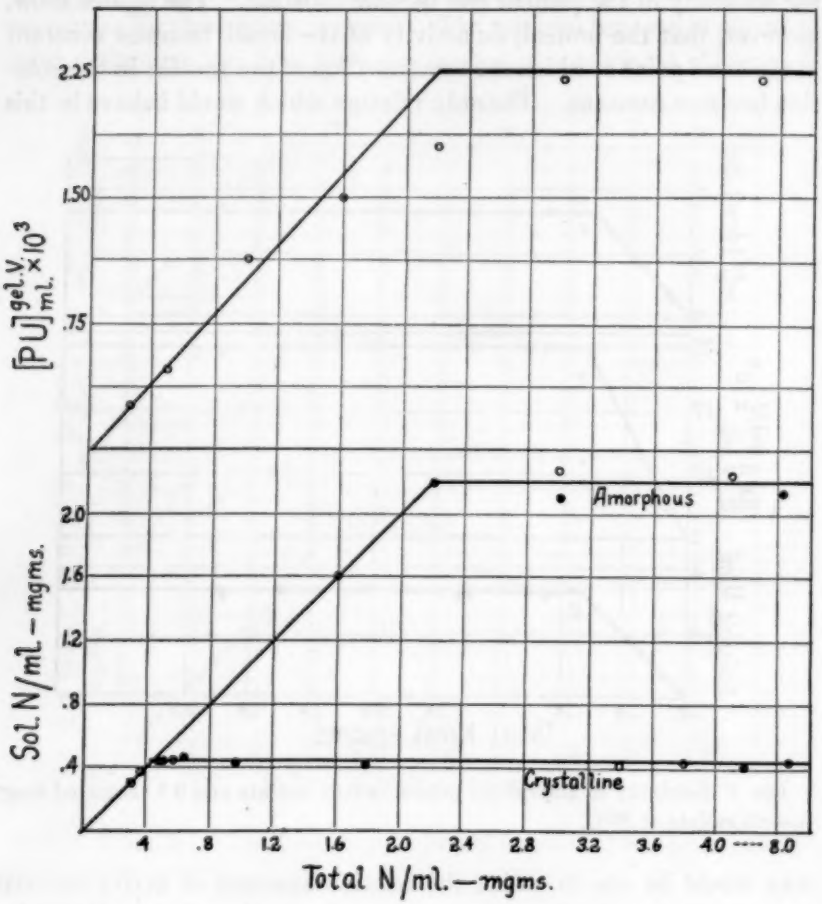

FIG. 5. Solubility of crystalline and amorphous pepsin in 0.055 acetate and 0.444 saturated magnesium sulfate at 20°C.

appears would differ, however, from the ratio in the original material. The ratio in the solution in that part of the curve where there is a large amount of solid present would also differ from the ratio in the original

material. The figures show that this latter condition does not occur. The activity per gram of nitrogen in the solution in equilibrium with a

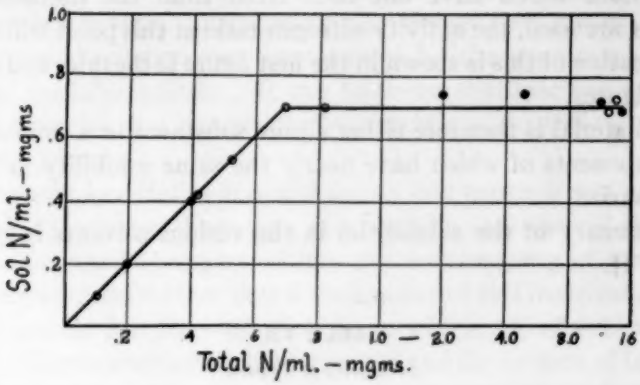

FIG. 6. Solubility of crystalline pepsin in 0.522 M sodium sulfate and 2.5 × 10^{-5} N sulfuric acid at 20°C.

TABLE VI

$$Ratio\ \frac{Activity}{Nitrogen} = [PU]^{\frac{gel.\ V.}{gm.\ N}}$$

Experiment	Pepsin	Solvent	In precipitate when nearly all soluble	In solution when large excess of solid present	In original crystals
26	Crystals	0.05 acetate pH 4.65, 0.50 sat. $MgSO_4$	0.75 1.10	1.00 1.09	1.11 ± 0.05
23	"	" " " " " " "	1.2	1.11	
22	Amorphous	" " " " " " "	1.20	1.16	
21	"	0.01 " " " " " "	1.09	1.02 1.13	
20	"	" " " " " " "	1.11	1.21	

large excess of precipitate is the same within the experimental error as the ratio in the original material. The activity-nitrogen ratio of the

first precipitate to appear is also the same in the experiments with amorphous and crystalline material as is shown in Table VI. If old preparations which have not been freed from the decomposition products are used, the activity-nitrogen ratio at this point will be low. An indication of this is shown in the first figure in the table and in some of the figures.

The material is therefore either a pure substance or a solid solution the components of which have nearly the same solubility in all the solvents tried.

A summary of the solubilities in the various solvents is given in Table VII.

TABLE VII

Solubility of Pepsin

Preparation	Solvent	Temperature	Solubility N/ml.
			mg.
Crystals	0.522 M Na_2SO_4, 2.5×10^{-5} N H_2SO_4	20°	0.70
"	0.444 M Na_2SO_4, 0.0556 acetate buffer pH 4.65	20°	.43
Amorphous	" " " " " " "	20°	2.2
Crystals	0.05 acetate pH 4.65, 0.50 sat. $MgSO_4$	8°	.22
Amorphous	" " " " " " "	20°	1.00
"	0.01 " " " " " "	20°	.65

Inactivation.—It was pointed out by Pekelharing that the temperature coefficient for the inactivation of enzymes and especially of pepsin

was extremely high and agreed with that for the denaturization of proteins. No other reaction known has such an enormous temperature coefficient and the agreement of the two values suggests that the enzymes are proteins. Pekelharing also showed that loss of activity in strong acid solution was accompanied by the appearance of denatured, insoluble protein. It can be shown that inactivation either by heat or by alkali is quantitatively proportional to the denaturization of the protein.

Inactivation by Alkali.—It is well known that pepsin is very sensitive to alkali. Goulding, Wasteneys and Borsook (13) showed that this inactivation could be separated into two reactions, one of which was instantaneous; and further that if the amount of this inactivation were plotted against the pH a titration curve with a pK of 6.85 was obtained. These experiments were repeated and the amount of inactivation compared with the amount of denatured protein found.

Experimental.—A series of 1 per cent solutions of the pepsin was made and adjusted to about pH 5. Increasing amounts of alkali were then added to these solutions so as to give a series of solutions varying in pH from about 5.5 to 9. An amount of acid equivalent to the amount of alkali in the tube, was added immediately after the addition of the alkali and the activity of the resulting solution determined after neutralization. 5 ml. portions were added to 5 ml. of a solution of half-saturated sodium sulfate and M/500 sulfuric acid. The unchanged pepsin is soluble under these conditions (to about 1 per cent) but the denatured protein is insoluble. The denatured protein therefore precipitates and the unchanged protein nitrogen is determined from the filtrate. In this way the percentage inactivation and percentage denaturization may be determined. The denatured protein is rapidly digested by the active enzyme so these solutions must be precipitated and filtered as soon as possible.

Control experiments with known mixtures of denatured and active pepsin show

that very little activity is carried down with this precipitate if the activity is determined by hydrolysis of casein. If the activity is determined by hydrolysis of gelatin, however, the precipitate of denatured protein may be quite active; and this result is evidently due to the abnormal activity observed throughout in relation to gelatin hydrolysis. This complication may be avoided by determining the activity of the solution before precipitation.

The results of such an experiment are shown in Fig. 7. The inactivation of the pepsin is quantitatively parallel with the denaturization of the protein since the percentage of activity left is the same as the

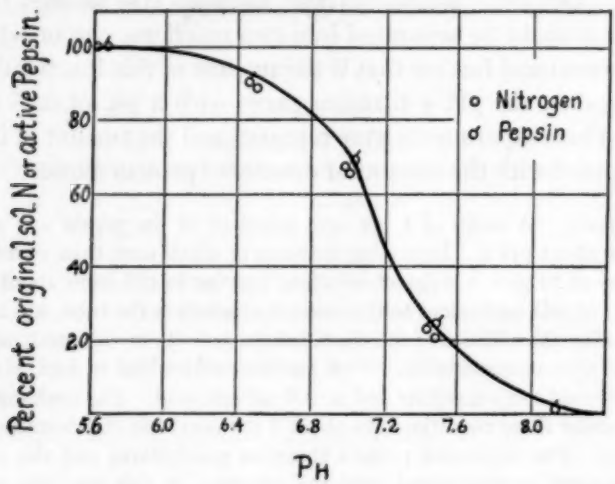

FIG. 7. Percentage inactivation and percentage denaturization of pepsin at various pH at 20°C.

percentage of soluble nitrogen remaining. The curve is very similar to that of Wasteneys and Borsook but is a little more to the alkaline side.

Inactivation by Heat.—Pekelharing noted that when pepsin solutions were allowed to stand in a range of acidity where the pepsin is active, the protein was digested and at the same time the activity of the solution decreased. He concluded from this that the pepsin digested itself. It can easily be shown, as was mentioned above, that the denatured protein is rapidly digested and it seems probable that the effect noticed by Pekelharing was the result of denaturization of the protein followed by hydrolysis. It is then unnecessary to suppose that the enzyme di-

gests itself. Pepsin solutions when heated slowly or when maintained at a temperature at which the inactivation proceeds slowly, do not contain denatured, insoluble protein since the latter is hydrolyzed as rapidly as it is formed. Under these conditions inactivation of pepsin is accompanied by the appearance of soluble nitrogen instead of insoluble; and it is difficult to make a sharp separation of the original pepsin from the products of hydrolysis. The result of an experiment which shows the digestion of denatured pepsin by active pepsin is given in Table VIII in which mixtures of various amounts of active and denatured pepsin were made up at pH 3 and left at 35°C. for 24 hours.

TABLE VIII

Digestion of Inactivated Pepsin pH 3.0, 35°C.

Pepsin dissolved in HCl, pH 3.0 = Solution A
50 ml. A boiled, cooled = Solution D
A and D mixed in various proportions,
2 ml. sample of mixture taken and
2 ml. M/1 Na_2SO_4 added, filtered,
N/ml. determined in filtrate.

A	20	10	5	0
D	0	10	15	20
Mg. soluble N in filtrate after:				
0 hrs.	.30	.11	.07	0
20 "	.30	.30	.28	.01

The denatured pepsin is precipitated by M/1 Na_2SO_4 at pH 3, while the active pepsin is soluble. The table shows that the inactive and insoluble pepsin is dissolved by the active pepsin.

It may be shown, however, by means of solubility experiments, that the loss of inactivation in the solution is exactly paralleled by the disappearance of the original protein. If a saturated solution of pepsin in M/300 hydrochloric acid is placed at 65°C. the activity is rapidly lost so that only about half the original activity remains after 15 minutes. No precipitate occurs but the formol titration increases. If the solution is now cooled and stirred again with a large quantity of crystalline pepsin more of the crystals must go into solution to

replace the material which has been changed. If the inactivation of the enzyme is accompanied by some chemical change in the protein then the amount of material which will dissolve must be proportional to the percentage loss in activity. That is, if half the activity is lost the increase in solubility must be equal to half the original solubility (assuming the solubility to be unaffected by the presence of the inactivated enzyme). On the other hand, the activity per milliliter of the solution after being stirred with the solid should return to its original value. The result of an experiment designed to test this assumption is shown in Table IX. The solution was heated for 15 minutes at 65°C. and in that time the activity per milliliter had been reduced to 50 per cent of its original value, whereas the formol titration

TABLE IX

Inactivated Pepsin and Solubility. 65°C.

	$[PU]^{gel.\ V.}_{ml.} \times 10^3$	N/ml.	Formol/ml.
		mg.	*ml. 0.01 NaOH*
M/300 HCl, stirred 1 hr. with pepsin, 20°C. Centrifuge	0.315	0.30	0.22
Supernatant heated at 65°C. for 15 min.	.160	.30	.33
Pepsin crystals added and stir 1 hr. Centrifuge			
Observed	.325	.45	.45
Calculated	.315	.455	.44

had increased 50 per cent and the total N per milliliter was unchanged since no precipitate appeared. The solution was then cooled and stirred at 20°C. with more crystals. The suspension was then filtered and the supernatant analyzed. The activity per milliliter is now the same as that of the original solution while the nitrogen per milliliter is 50 per cent higher than the original value. The formol titration has been increased by 50 per cent of its original value also. The results show that the loss of activity is exactly paralleled by some chemical change in the protein molecule.

The Rate of Inactivation at 65°C.—It has frequently been found that the rate of inactivation of enzymes does not follow the course of a simple monomolecular reaction but becomes slower as the reaction

proceeds. It was found by the writer (6) that this peculiarity was connected with the purity of the enzyme solution and that the rate of inactivation of more highly purified solutions agreed with the monomolecular reaction rate, while those solutions containing inhibiting substances did not. On this basis the rate of inactivation of the crystalline material would be expected to be monomolecular. The result of an experiment in which the rate of inactivation of a 0.06 per cent solution of pepsin in 0.001 M hydrochloric acid (pH 3) at 65°C. is shown in

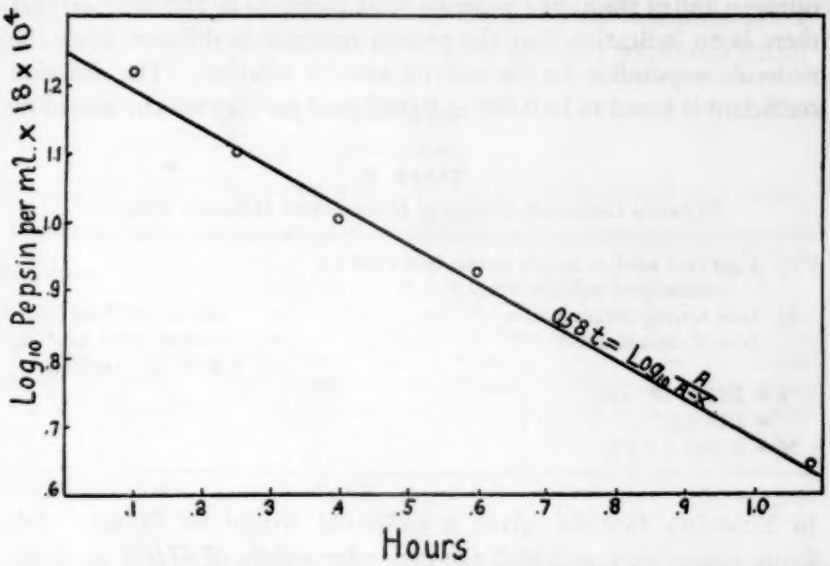

FIG. 8. Rate of inactivation of 0.06 per cent pepsin in 0.001 M hydrochloric acid at 65°C.

Fig. 8. The reaction follows the theoretical monomolecular course quite closely.

Identity of the Diffusion Coefficient of the Protein and of the Active Material.—The preceding experiments have shown that the activity cannot be separated from the protein molecule either by solubility measurements or inactivation experiments. It might be assumed, however, that the crystalline substance was analogous to a double salt and that the active material and the protein were in equilibrium with each other but were separate in solution. If this were the case it would

be expected that the rate of diffusion of the two would be different. The diffusion coefficients were therefore determined by the method previously described (14). The experiments were carried out at 8°C. and the amount of material diffusing was determined by analyzing for nitrogen and also by activity determinations. The results of this experiment are shown in Table X. The diffusion coefficient was found to be the same when determined by either nitrogen or by activity. The radius (and presumably the weight) of the molecule containing the nitrogen and of the active molecule must therefore be the same, so that there is no indication that the protein molecule is different from the molecule responsible for the activity even in solution. The diffusion coefficient is found to be 0.048 ± 0.0005 cm.² per day which, according

TABLE X

Diffusion Coefficient, Radius of Molecule and Molecular Weight

8°C.	1 per cent solution in M/1 acetate buffer pH 4.6, viscosity of buffer = 0.017.	
D	from activity determination	= .047 ± .0005 cm.²/day
	from N determination	= .0468 ± .0005 cm.²/day
		= 5.44×10^{-7} cm.²/sec.
r	= 2.22×10^{-7} cm.	
	= 2.22 mμ	
M	= 37,000 ± 1,000	

to Einstein's formula, gives a molecular weight of 36,000. This figure agrees very well with the molecular weight of 37,000 as determined by osmotic pressure measurements. The agreement between these two figures shows that the molecules must be nearly all the same size since the molecular weight gives a mean value while the diffusion experiment as carried out gives a minimum value. The agreement also shows that the molecule is not hydrated to any great extent, since the value used for the specific gravity in calculating the molecular weight from the diffusion coefficient is the specific gravity of dry protein; and the value for the molecular weight so obtained agrees with that found by osmotic pressure.[3]

[3] Preliminary experiments (15) gave larger diffusion coefficients corresponding to a molecular weight of about 10,000, but this result was found to be due to an error in calibrating the diffusion cell.

Antipepsin.—If the enzyme is a protein an antibody should be formed on injection into an animal. The production of such antienzymes has been reported in the literature but the results are uncertain, as Euler has pointed out (16), because of lack of information in regard to the activity of the preparation used. In order to see if any antibody could be produced and if its production were associated with the activity of the preparation, two rabbits were immunized, one with an active preparation of the enzyme and one with a preparation of the same concentration which had been inactivated by alkali. 5 ml. injections of a 1 per cent solution of the active or inactive material were made intra-

TABLE XI

Antiserum

1 ml. 1 per cent pepsin solution in M/5 4.6 acetate buffer, diluted with buffer, + 0.15 ml. serum.

Pepsin dilution		1	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,000	1/2,000	0
Pepsin	Serum	0 serum	.15 ml. serum												
Active	Active	−	++	++	++	++	++	+	+	+	+	+	+	+	−
"	Denatured	−	++	++	++	++	+	−	−	−	−	−	−	−	−
"	Normal	−	−	−	−	−	−	−	−						
Denatured	Active	−	++	++	+	+	+	−	−	−	−	−	−	−	−
"	Denatured	−	++	++	++	+	−	−	−	−	−	−	−	−	−
"	Normal	−	−	−	−	−	−	−	−	−	−	−	−	−	−

peritoneally at intervals of about 1 week. Four injections were made; the rabbits were bled 2 weeks after the final injection and the serum obtained in the usual way. These sera gave a precipitin reaction with the pepsin solution, as shown in Table XI. Normal serum gave no precipitate with either the active or the inactive pepsin preparation. The serum obtained from the rabbit injected with active pepsin gave a positive precipitin reaction in a dilution of about 1/2,000 with the active pepsin solution and to about 1/16 with the inactive solution. The serum from the rabbit immunized with denatured pepsin gave a precipitin test with the denatured preparation in a dilution of about 1/8 per cent solution and with the active preparation to

about the same extent. Both sera gave precipitin reactions with a 1 per cent solution of pepsin inactivated by boiling in a dilution of about 1/16. As might be expected, therefore, the sera were not strictly specific but gave cross precipitations with both active and denatured pepsin. The inhibiting effect of the sera on the activity was tested by mixing increasing amounts of the antiserum with a small amount of active pepsin and determining the rate of digestion of casein or gelatin with this mixture. The result of this experiment is shown in Table XII.

TABLE XII

Effect of Antiserum on Digestion of Casein or Gelatin by Pepsin

Serum diluted as noted + M/5 4.6 acetate buffer. Pepsin in 4.6 acetate buffer. 1 ml. pepsin solution + 1 ml. serum dilution.

Gelatin

Serum	Final dilution of serum					
	1/4	1/8	1/16	1/32	1/64	0
	$[PU]^{gel.\ V.}_{ml.}$ after 3 hrs. × 10^3					
Normal	0.075	0.10	0.11	0.12	0.11	0.12
Antidenatured	.060	.10	.09	.10	.11	.12
Antiactive	.045	.075	.10	.10	.11	.12

Casein

Serum	0	Normal	Anti-denatured	Antiactive
Soluble N in filtrate, mg.	2.2	2.0	1.87	1.87
$\propto [PU]^{cas.\ S.}_{ml.}$	.015	.008	.007	.007

Both the serum prepared by the injection of the active pepsin and that prepared by the injection of the denatured pepsin inhibit the action of the enzyme to about the same extent when tested by the solution of casein (cas. S. method). The normal serum inactivates somewhat less although there is no very great difference. It is difficult to compare the action of the various sera quantitatively from this experiment. The effect may be seen more clearly by determining the rate of hydrolysis of gelatin (gel. V. method) with a small amount of

pepsin to which has been added increasing amounts of the various sera. The result of such an experiment is also shown in Table XII. The active serum inhibits quite strongly in a dilution of 1:4, the denatured serum less so and the normal serum still less. This inhibiting effect is evidently very weak compared with that of many antitoxins, although the precipitin reaction is about what would be expected. This may be due to the fact that the pepsin injected must be almost instantly inactivated since, as was shown above, the pepsin is instantly more than half inactivated at a pH of 7. It is probable therefore that the active pepsin is in the circulation of the animal for only a very short time.

The writer is indebted to Dr. F. S. Jones and Dr. A. P. Krueger for advice and assistance in connection with these experiments.

VI

Conclusions as to the Purity of the Preparation

The preceding experiments have shown that no evidence for the existence of a mixture of active and inactive material in the crystals could be obtained by recrystallization, solubility determinations in a series of solvents, inactivation by either heat or alkali, or by the rate of diffusion. It is reasonable to conclude therefore that the material is either a pure substance or a solid solution of two very closely related substances. If it is a solid solution of two or more substances it must be further assumed that these substances have about the same degree of solubility in the various solvents used, as well as the same diffusion coefficient and rate of inactivation or denaturization by heat. It must also be assumed that both substances are changed by alkali at the same rate and to the same extent. This could hardly be true with the possible exception of two closely related proteins. It is conceivable that two proteins might be indistinguishable by any of the tests applied in this work. But in this case it would follow that the enzyme itself was a protein and this, after all, is the main point. It does not necessarily follow even if the material represents the pure enzyme that it is the most active preparation that can be obtained nor that it is the only compound which has proteolytic activity. There is some evidence that the activity of the preparation may depend on its physical state as is known to be the case with the catalytic activity of

colloidal metals. It is possible, on the other hand, that hemoglobin is the type structure for the enzymes and that they consist of an active group combined with a protein as suggested by Pekelharing. The active group may be too unstable to exist alone, but it is quite conceivable that a series of compounds may exist containing varying numbers of active groups combined with the protein, and that the activity of the compound would depend on the number of these active groups. This hypothetical complex would not differ much from that assumed by Willstätter (18) and his coworkers, except that it supposes a definite chemical compound with the protective group in place of an adsorption complex. It is of course possible that both types of complex may be formed under suitable conditions. The reactivation of enzymes as reported in the literature also suggests their protein nature since the conditions for this reactivation are similar to those found by Anson and Mirsky (20) to be suitable for the formation of native from denatured protein. The fact that the crystalline urease prepared by Sumner is also a protein and that the temperature coefficient for the rate of inactivation of enzymes in general is that characteristic for the denaturization of proteins, suggests that the protein fraction in the purification of enzymes be given special attention even though it may not be the most active fraction.

The crystalline pepsin resembles the amorphous preparations obtained previously by Pekelharing, Ringer, Fenger, Andrew and Ralston and other workers. It is probable that the preparations obtained by these workers were nearly pure pepsin. Both Pekelharing and Ringer (17) obtained preparations free from phosphorus so that there may be several proteolytically active forms.

Pekelharing showed that the same protein material could be obtained from gastric juice as from autolyzed gastric mucosa so that it is probable that the crystalline material could also be readily prepared from gastric juice. It seems fair to conclude therefore that the crystalline protein described in this paper is identical with the enzyme pepsin as secreted by the animal.

VII

Proteolysis Is a Homogeneous Reaction

Sørensen has shown that a solution of egg albumin is a one-phase system and the solubility measurements described in this paper show that a solution of pepsin consists also of only one phase. The reaction between dissolved egg albumin and dissolved pepsin therefore is a case of homogeneous, rather than heterogeneous catalysis.

SUMMARY

A method is described for the preparation of a crystalline protein from commercial pepsin preparations which has powerful peptic activity. The composition, optical activity, and proteolytic activity of this protein remain constant through seven successive crystallizations. No evidence for the presence of a mixture or of a solid solution is found in a study of the solubility of the protein in a series of different salt solutions, nor from the diffusion coefficient or from the rate of inactivation. These results indicate that the material is a pure substance or possibly a solid solution of two or more substances having nearly the same solubility in all the various solvents studied. It seems reasonable to conclude from these experiments that the possibility of a mixture must be limited to a mixture of proteins, so that the conclusion seems justified that pepsin itself is a protein.

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CRYSTALLINE PEPSIN

II. General Properties and Experimental Methods

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The isolation of a crystalline protein having proteolytic activity was described in the preceding paper (1). The results of solubility, inactivation, and diffusion experiments showed that there was reason to believe that the preparation was a pure substance and that the peptic activity was a property of the protein molecule itself. The following paper contains some further properties of the pepsin and a description of the experimental procedure.

Solubility in Various Concentrations of Sodium Sulfate.—The solubility in various concentrations of sodium sulfate is shown graphically in Fig. 1. A concentrated suspension of the crystalline material was made in sodium sulfate of the required concentration made up in 0.001 M sulfuric acid. These suspensions were stirred at 35°C. for 10 minutes and then cooled to 25°C. for 24 hours. The solubility was therefore reached from the supersaturated side. There is a sharp maximum of solubility at about 0.1 M sodium sulfate.

Solubility at Different Temperatures.—The solubility in 0.50 saturated $MgSO_4$ and 0.05 acetate pH 4.65 at various temperatures is shown in Fig. 2. The solubility increases slightly but there are no sharp breaks in the curve and therefore no indication that the material is a double salt, nor that different solid phases appear at different temperatures.

Solubility in Various Concentrations of Hydrochloric or Sulfuric Acid.—These determinations were made by stirring a concentrated suspension with various concentrations of acid as described for the sodium sulfate experiments. The results are given in Fig. 3. There is a minimum of solubility at about pH 2.75 corresponding presumably to the isoelectric point. The solubility increases rapidly on both sides of

this point. The increase in solubility on the acid side cannot be ascribed to salt effect since the series represented by triangles was made with a mixture of hydrochloric acid and sodium chloride in which the

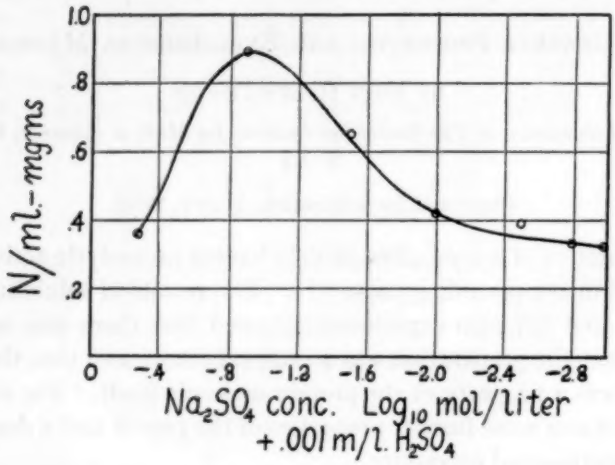

FIG. 1. Solubility of crystalline pepsin in various concentrations of sodium sulfate made up in 0.001 M hydrochloric acid at 25°C.

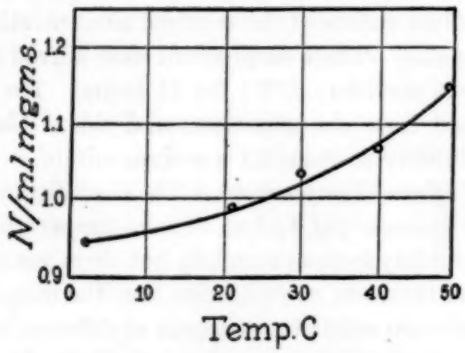

FIG. 2. Solubility of crystalline pepsin in 0.5 saturated magnesium sulfate and 0.05 M acetate at various temperatures.

chloride concentration was kept constant throughout. When the solubility is plotted against the concentration of acid instead of the pH the solubility increases in direct proportion to the addition of acid

and each millimole of acid dissolves about 0.05 mg. of nitrogen, corresponding to 0.33 mg. of pepsin. This is the expected relation for a

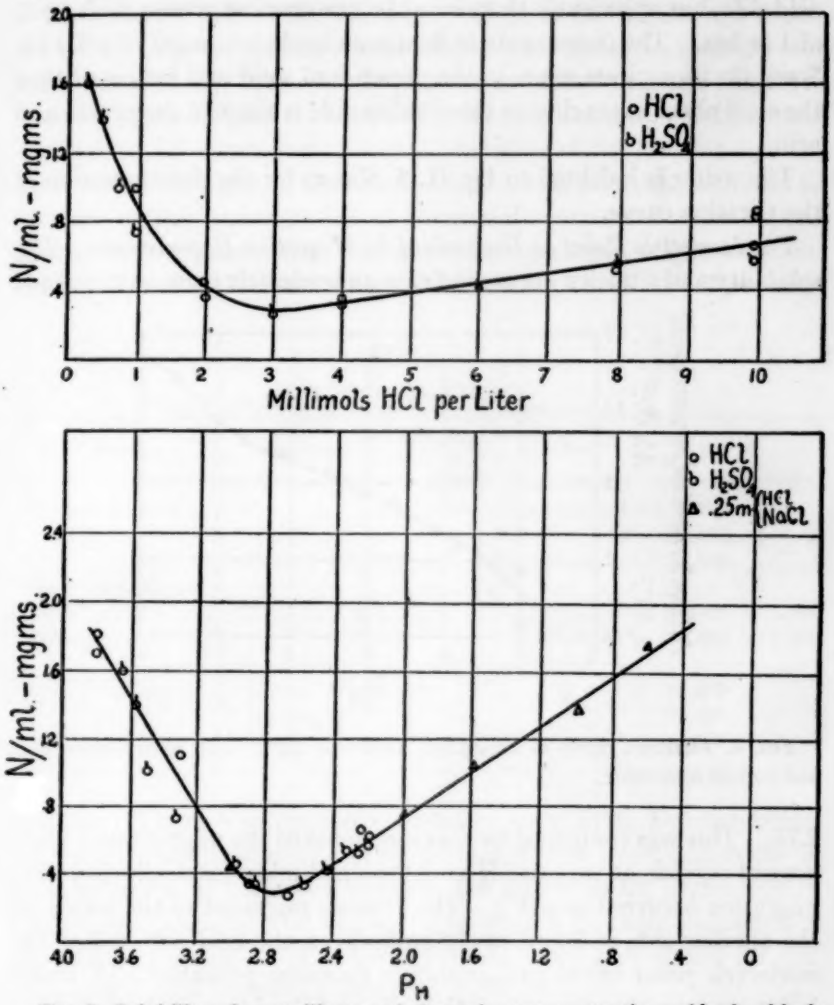

FIG. 3. Solubility of crystalline pepsin in various concentrations of hydrochloric or sulfuric acid.

monovalent base (2). On the alkaline side of the minimum the solubility curve is convex downward. This is the theoretical shape for the solubility curve of a polyvalent acid.

Titration Curve.—The titration curve of the pepsin is shown in Fig. 4. Owing to its insolubility the curve could not be followed on the acid side; but apparently there must be one or more groups with a pK of 1 or less. The curve again indicates an isoelectric point of pH 2.75. Some titrations were made in the presence of solid and indicated that the solid phase appearing on the alkaline side is a salt of the pepsin and acid.

The writer is indebted to Dr. H. S. Simms for the determination of the titration curve.

The Isoelectric Point as Determined by Migration Experiments.—The solubility and titration curves indicate an isoelectric point at about pH

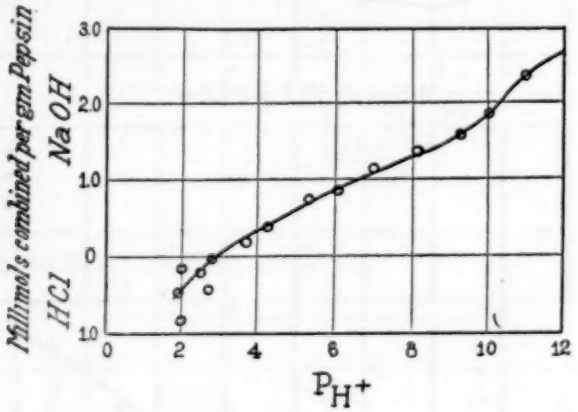

FIG. 4. Titration curve of crystalline pepsin at 22°C. with hydrochloric acid and sodium hydroxide.

2.75. This was confirmed by measurements of the migration of finely ground crystals at various pH as determined microscopically (3). No migration occurred at pH 3. The crystals migrated to the anode on the alkaline side of 3 and to the cathode on the acid side of 3. The isoelectric point of the preparation is therefore probably 2.75, which is not far from that found by Michaelis and Davidson (4) in a study of the migration of crude pepsin preparations. Ringer (5) had, however, stated that some pepsin preparations were always negatively charged and the writer (6) had found that the distribution of pepsin with gelatin particles also indicated that the enzyme was always negatively

charged on the alkaline side of pH 2.0. It does not seem possible at present to account for these discrepancies.

Molecular Weight.—The molecular weight of the pepsin was determined by osmotic pressure measurements in molar sodium acetate buffer at pH 4.60. Since this is quite far from the isoelectric point it is necessary to show that the observed pressure is due to the protein itself and not to a Donnan equilibrium. The effect of the Donnan equilibrium would be slight in molar salt solution and would be expected to increase as the concentration of the protein increased. The experiments, however, show that the calculated molecular weight was constant within the experimental error for a 4 per cent and a 2 per cent

TABLE I

Molecular Weight

Method	Temperature	Concentration of solution	Solvent	M
Osmotic pressure	8°	4 per cent	M/1 acetate pH 4.6	34,000
			" " "	33,000
	8°	2 " "	" " "	36,000
Diffusion coefficient				
Activity	8°	1 " "	" " "	37,000 ± 1,000
Nitrogen			" " "	36,000 ± 1,000
From P content	1 atom P per mole			40,000
Cl "	2 atoms Cl " "			35,000
S "	10 " S " "			36,000

solution. There was probably therefore very little Donnan effect. The molecular weight may also be calculated from the diffusion coefficient by means of Einstein's formula, and also from the percentage of phosphorus, chlorine and sulfur. The results of these calculations are shown in Table I. The various methods all give values for the molecular weight between 33,000 and 38,000.

Experimental Procedure

Material.—The starting point of this work was Parke, Davis pepsin U. S. P. 1:10,000. This material has already been highly concentrated from autolyzed pig stomachs. According to information kindly furnished by Mr. F. O. Taylor, Chief

Chemist of Parke, Davis and Company, the method of purification is in general that of Davis and Merker (7).

Rate of Hydrolysis of Casein, Egg Albumin, Edestin or Gelatin as Determined by the Increase in Carboxyl Groups

The egg albumin had been crystallized three times. Hammersten's casein (Kahlbaum) was used. The edestin was prepared from hemp seed and had been recrystallized twice. The gelatin was prepared from Cooper's powdered gelatin as previously described (8).

The protein solutions were titrated to the required pH with HCl and diluted so as to contain 5 gm. protein per 100 ml. They were then heated to boiling to denature the proteins and stored at 5°C. 5 ml. of the protein solution are pipetted into a series of 50 ml. centrifuge tubes and placed in a water bath at 35.5°C. 1 ml. of the pepsin solution is added to each tube. After about 2, 4, 6 and 8 minutes 5 ml. of 0.10 NaOH and 1 ml. of formalin are added to each tube. They are then titrated to pH 10 with N/50 NaOH (9). The increase in the alkali needed over that used for a control tube containing 1 ml. boiled pepsin solution is the titration. These figures are then plotted against the time of hydrolysis and the increase in the formol titration for the first minute interpolated from the curve. The proteolytic activity per gram is equal to this amount, expressed as milliequivalents divided by the grams dry weight per milliliter of the pepsin solution used.

$$i.e.,\ [PU]_{gm.} = \frac{\text{ml. N/50 NaOH} \times 20 \text{ per minute}}{\text{mg. pepsin per ml.}}$$

The determination is quite accurate with casein or edestin but in the case of egg albumin and gelatin the rate of hydrolysis decreases rapidly after an increase of only 0.3 to 0.5 ml. N/50 NaOH, so that interpolation is difficult unless very small titrations are used and in this case the experimental error of the titration is large.

Determination of Activity by Comparative Methods

The activity determined in this way probably represents the most rational measure of the rate of hydrolysis. For routine comparative purposes, however, it is not convenient since it requires a number of analyses for each determination and since the experimental error is rather large. After the activity in rational units has been determined by this method, however, it is possible to use comparative methods which are more accurate and also more convenient. In order to do this a solution the activity of which has been determined by the previous method is used as a standard. Several comparative methods of this kind have been used.

Increase in Formol Titration after 1 Hour at 35°C. (cas. F.), (gel. F.), etc.—Increasing amounts of the standard pepsin solution containing a known number of PU as determined by the rate of liberation of carboxyl groups as described above, are

added to 5 ml. portions of the various protein solutions and the hydrolysis allowed to proceed for 1 hour at 35.5°C. At the end of this time the increase in the formol titration is determined as described above. This increase is then plotted against the number of proteolytic units added so as to give a standard curve. The activity of an unknown solution is then determined by carrying out the hydrolysis in the same way and interpolating the value of the activity from the standard curve.

Solution of Casein (cas. S.).—1 ml. of various dilutions of the standard pepsin solution is added to 5 ml. of 5 per cent casein pH 2 and kept at 35.5°C. for 21 hours. 5 ml. of this solution is then pipetted into 5 ml. of 20 per cent trichloracetic acid, filtered and the total nitrogen determined in 5 ml. of the filtrate. A standard curve is plotted from these figures and the activity of the unknown solution found by interpolation from this curve. This is the most accurate method, the probable error of a single determination being less than 2 per cent.

Rennet Action (R).—The amount of pepsin solution which is just sufficient to solidify 5 ml. of milk at 35.5°C. in 2 hours is determined. This figure cannot be compared directly with the activity as determined by the liberation of carboxyl groups. For purposes of comparison therefore it is arbitrarily assumed that the activity of the standard solution of crystalline pepsin is the same with respect to rennet action as with respect to the hydrolysis of casein as determined by the rate of liberation of carboxyl groups. 0.085 mg. crystalline pepsin equivalent to 0.008 $[PU]^{cas.\ F.}$ coagulates 5 ml. of milk under the above conditions. 1 ml. of a solution which causes coagulation of 5 ml. of milk in 2 hours therefore contains 0.008 $[PU]^{R}$. The time varies greatly with different samples of milk so that it is necessary to run a known solution simultaneously with the unknown.

Gelatin Liquefaction Method (10) pH 5 (gel. V.).—0.2 ml. of the standard pepsin solution is added to 5 ml. of 2.5 per cent solution of isoelectric gelatin in water and the mixture poured into a viscosimeter at 35.5°C. The change in viscosity is then determined at intervals and the time of outflow after various time intervals plotted against the elapsed time since mixing. The time required to cause a 3 per cent change in the original viscosity is then interpolated from these curves. The time required to cause this change is inversely proportional to the quantity of pepsin so that the activity of an unknown solution in terms of the standard is found by proportion. Since there is no simple relation between the rate of change in viscosity and the liberation of carboxyl groups (11), it is assumed arbitrarily that the activity of the standard crystalline pepsin solution is that determined by the rate of liberation of carboxyl groups from pH 2.5 gelatin. Under these conditions 0.14 mg. of crystalline pepsin equivalent to 2.4×10^{-5} $[PU]^{gel.\ F.}$ cause a 3 per cent change of viscosity in 0.30 hours. Since the activity is inversely proportional to the time

$$[PU]^{gel.\ V.}_{0.2\ ml.} = \frac{0.72 \times 10^{-5}}{t\ hrs.}$$

and

$$[PU]^{\text{gel V.}}_{\text{ml.}} = \frac{3.6 \times 10^{-5}}{t \text{ hrs.}}$$

where t is the time in hours for 0.2 ml. of the unknown solution to cause a change of 3 per cent in the viscosity of the standard gelatin solution.

Nitrogen Determination.—The nitrogen was determined by a slight modification of the Folin and Farmer (12) micromethod using the following digestion mixture:

2 ml. concentrated H_2SO_4
1.5 gm. K_2SO_4
0.2 gm. mercuric oxide

Digestion is continued 20 minutes after the solution is colorless in 50 ml. Kjeldahl flasks having a constriction half-way up the neck to prevent loss of acid.

35 ml. H_2O, 0.2 gm. sodium hypophosphite and 10 ml. of saturated NaOH are added and the solution distilled through two Kjeldahl traps into standard N/50 acid. 2 to 5 mg. of nitrogen are taken for a determination. The method is accurate to about 0.2 per cent provided the double trap is used. Otherwise there are irregular variations due to spattering of the alkali.

P Determination.—The excellent method described by M. Sørensen (13) was used.

Experimental Methods for the Solubility Determinations

Crystals.—The first attempts to determine the solubility of pepsin were made in the usual way by stirring a suspension of the solid with the liquid and analyzing the supernatant fluid after various times. It was found, however, that equilibrium could not be obtained in this way except when a very large amount of the solid was present. Otherwise, the amount of material dissolved increased rapidly for the first 24 hours and gave what appeared to be an equilibrium value. This value, however, was different with different quantities of precipitate, being higher for the larger quantity. If the stirring were continued, however, it was found that the solubility continued to increase slowly in all the suspensions and this increase continued at a nearly constant rate, apparently indefinitely. At the same time the ratio of activity to nitrogen in the filtrates decreased and a form of nitrogen which could not be coagulated by heat began to appear in the solution. It was evident therefore that this slowly continued increase in soluble protein was not simply a process of solution but was due to the decompostition of the protein. Various methods of stirring combined with low temperatures were tried without success, although it was found that stirring with a glass ball in a test tube completely filled with solution gave much better results.

With large quantities of precipitate constant values for the solubility could be obtained very rapidly by stirring the suspension in a long narrow tube, the stirrer

consisting of a glass rod of about half the diameter of the tube so that there is only a thin layer of suspension between the stirrer and the walls of the tube. The stirrer was rotated at about 500 R.P.M. There is very efficient stirring under these conditions and constant values for the solubility were obtained after about 10 minutes, provided the amount of solid material was more than 3 or 4 times as much as the quantity in solution. The figure for the solubility under these conditions was found to be independent of the quantity of precipitate when relatively large amounts of precipitate were used. However, if the quantity of precipitate were small then equilibrium values could not be obtained before decomposition commenced, so that it was not possible in this way to obtain the part of the curve where there is very little solid. This portion of the curve, however, could be obtained from the supersaturated side. The method finally adopted for solubility measurements may be outlined as follows.

A suspension of freshly recrystallized protein was filtered by suction and washed on the filter 5 or 6 times with the solvent that was to be used for the solubility determinations. About 25 gm. of the filter cake were then suspended in 100 ml. of the solvent and stirred for 15 minutes at the desired temperature, the stirrer being arranged as described above. The suspension was then filtered and the nitrogen content of the filtrate determined. The precipitate was stirred again with 100 ml. of the solvent and this process was repeated until successive determinations gave the same value for the nitrogen in the filtrate. Aliquot portions of the suspension were diluted with solvent so as to give a series of suspensions containing 1/2, 1/4, 1/8 and 1/16 of the concentration of solid present in the original suspension. Each of these suspensions was then stirred for 15 to 20 minutes, filtered and the nitrogen, proteolytic activity and optical rotation of the filtrate determined. These figures represent the solubility as determined from the undersaturated side.

A second series of suspensions was made up in the same way and stirred at 45°C. for 5 to 10 minutes. A small sample of supernatant was taken at 45°C. and analyzed for nitrogen. The figure obtained in this way was always higher than that found at the lower temperature. These suspensions were then cooled to the original temperature and stirred occasionally. After 24 hours they were filtered and the supernatant analyzed as in the first series. This method gives the solubility figure from the supersaturated side. In this way the solubility could be determined for suspensions containing more than twice as much solid protein as dissolved protein. In this range the solubilities determined from the undersaturated side nearly always agreed with those from the supersaturated side showing that they were real equilibrium values. When less solid was present, however, this was not the case. The values from the supersaturated side were always higher than those from the undersaturated side and were frequently higher than those obtained with large quantities of solids from the supersaturated side. In other words, they showed that equilibrium was not obtained when only a small quantity of solid was present. Attempts to obtain equilibrium values by longer stirring resulted only in indefinite increase in solubility accompanied with inacti-

vation. The solubilities at the point where very little solid was present could be obtained, however, by diluting a supersaturated solution and then inoculating with small amounts of solid. About 50 ml. of the original heavy suspension were stirred at 45°C. and filtered at this temperature. The filtrate was then diluted so as to give a series of solutions containing from 0.5 mg. to 3 or 4 mg. of nitrogen per milliliter. A few drops of the heavy suspension were then added to this series of solutions and the solutions cooled to the original temperature. In those solutions containing less than the saturation concentration the added solid dissolved and the solution became perfectly clear. The more concentrated ones remained cloudy and in those which had been diluted very little more solid crystallized out. These solutions were filtered and analyzed after 24 hours in the same way as the other series.

In this way a series of points were obtained showing the solubility up to and just beyond the appearance of the solid phase. The values were reproducible and those obtained when very little solid was present usually agreed well with the values obtained by the methods described for the case when a large amount of solid was present. There still remains a very small portion of the solubility-concentration curve corresponding to the point where the amount of solid was about equal to the amount of material in solution, for which equilibrium values could not be obtained. Values obtained by inoculating the supersaturated solution were usually higher than those found for the rest of the curve. The values obtained from the undersaturated side were usually lower.

Amorphous.—The solubility of the amorphous material as obtained from the undersaturated side was determined in the same way as with the crystals by stirring the precipitate with the solvent. In order to obtain the value from the supersaturated side, however, it was necessary to dissolve the precipitate and then reprecipitate it instead of allowing a supersaturated solution to cool since under these latter conditions crystals appeared. The solvent solution was therefore made up in two parts, in one of which the amorphous material was readily soluble. 20 to 30 gm. of the washed crystals were dissolved in as small a volume as possible of this part of the desired solvent and then precipitated by addition of the other part of the solvent. The solution was stirred during the precipitation and the precipitating solution run in slowly so as to avoid local excess. The amorphous precipitate obtained in this way was redissolved in the first part of the solvent and precipitated again.

This procedure was repeated until the nitrogen content of two or more successive filtrates was the same. This was taken as the value for the solubility from the supersaturated side. The suspension was then filtered and aliquot portions of the precipitate stirred with about 10 ml. of the complete solvent for 10 to 15 minutes. These suspensions were then filtered and analyzed as with the crystalline material. In this way a series of points were obtained showing the solubility obtained with varying amounts of precipitate from the undersaturated side. In order to obtain the value from the supersaturated side with different amounts of solid present the

remainder of the precipitate was dissolved in varying amounts of the first half of the solvent, so as to give a series of solutions containing from 1 to 3 or 4 mg. of nitrogen per milliliter. The second half of the solvent was then added to this series while the solution was being stirred and the suspension filtered at once. If the solutions were filtered immediately these values agreed well with those from the undersaturated side, but if the solutions were allowed to stand the values were consistently lower in those solutions in which only a small amount of solid appeared. This was at first very puzzling and it was thought that it might represent an actual fractionation of the material. However, the properties of the precipitate obtained in this way were precisely the same as that of the original material so that there was no evidence for any fractionation. Microscopic examination of these precipitates, however, showed that they contained some crystalline material, and since the solubility of the crystalline material is much less than that of the amorphous this was evidently the explanation for the low solubility obtained under these conditions. When a large amount of amorphous precipitate is present the material crystallizing out would be immediately replaced by solution of more amorphous so that under these conditions the saturation value of the amorphous precipitate was obtained. When only a small amount of amorphous material was present, however, the loss of material due to crystallization could not be replaced rapidly enough by solution of the amorphous material and so low values for the solubility resulted. In confirmation of this it was found that the values for the solubility obtained by filtering those solutions having very little precipitate immediately after the appearance of the precipitate agreed with those obtained in solutions containing larger amounts of precipitate. It was also found that inoculation of the solutions with a few crystals gave still lower values for the solubility since in this case crystallization proceeded more rapidly.

Composition of the Solutions Used.—In order to carry out the precipitation of the amorphous material as described above, it is necessary to make up the complete solvent by adding together two different solutions. Measured volumes of the two separate parts of the solvent must be used instead of making the mixture up to a constant volume in order to avoid the difficulty of correcting for the volume of the original portion of the concentrated suspension taken. The composition of the solvent is therefore expressed as the ratio of the volume of one portion to the total volume assuming that the volumes are additive. The value given in this way does not correspond strictly with the actual composition of the final solution but does define the composition of the complete solvent. For instance, a solvent designated as half-saturated magnesium sulfate and 0.05 M acetate buffer pH 4.65 refers to a solution prepared by mixing together equal volumes of saturated magnesium sulfate and 0.10 M acetate buffer pH 4.65. The saturated magnesium sulfate was prepared by stirring crystalline magnesium sulfate with water at about 18°C. The supernatant was filtered off and found to have a specific gravity at 22°C. of 1.2955 compared to water at that temperature. The sodium sulfate solution had a specific gravity at 22°C. of 1.120 and was 1.104 molar with respect to Na_2SO_4.

TABLE II

Summary of Properties of Pepsin

Crystalline form. Dihexagonal bipyramids, colorless, 0.01 to 0.1 mm. long. Positive double refraction.
Density. 1.32 gm. per ml.

$[\alpha]_D^{25°}$ (pH 4–5) −70°

Analysis. Per cent of dry weight.	Total N	Amino N	C	H	Cl	S	P	Ash	$SO_4^{=}$
	15.3	.80	52.4	6.67	.22	.86	.078	.40	00

Xanthoproteic and Biuret test—positive
Molisch test —negative

Diffusion coefficient 8° (M/1 acetate buffer, pH 4.6, Viscosity .017)
.047 ± .0005 cm.²/day (From N determination)
.0468 + .0005 " " (" activity determination)

Molecular weight from osmotic pressure (M/1 acetate pH 4.6)	35,000 ± 1,000
From diffusion coefficient	36,000 ± 1,000
From P content, 1 atom P per mole	40,000
Cl " 2 atoms Cl per mole	35,000
S " 10 " S " "	36,000

TABLE II (*Continued*)

Solubility	Solvent	.002 HCl	.522 M Na_2SO_4 2.5×10^{-6} N H_2SO_4	.444 M Na_2SO_4 .055 acetate pH 4.65	0.5 sat. $MgSO_4$ .05 acetate pH 4.65	0.5 sat. $MgSO_4$ .01 acetate pH 4.65
Mg. N/ml.	Crystalline	.3 (20°)	.70 (20°)	.43 (20°)	.22 (8°)	
	Amorphous			2.2 (20°)	1.00 (20°)	.65

Velocity constant inactivation

65°— pH 3.0 0.58 Time in hrs. Log_{10}.

1.98 per cent solution in HCl

Activity		Equivalents per mole pepsin per min. 35.5°C.
Substrate	pH	
Edestin	2.0	1,000
Casein	2.0	500
Denatured egg albumin	2.2	320
Gelatin	2.5	6.0

The acetate buffers were made up according to Walpole's curve (14). The pH values given are for solutions corresponding to 0.2 M acetate buffer and do not represent actual pH values of the final solution.

The analytical work and the preparation of most of the crystalline pepsin was carried out by Mr. N. Wuest.

SUMMARY

A number of properties of crystalline pepsin have been determined and are summarized in Table II.

The experimental procedure used is described.

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SOLUBILITY CURVES OF MIXTURES AND SOLID SOLUTIONS

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Theoretical Solubility Curves of Mixtures or Solid Solutions of Two Components

The isolation and characterization of biological compounds are attended with special difficulty since some substances, such as enzymes and vitamins, are active in extremely small amount while others, such as the proteins, differ so slightly in either chemical or physical characteristics as to render proof of their purity a difficult problem. It is frequently impossible to decide from analytical results whether the material is a mixture or a solid solution or a pure substance. The phase rule offers the most powerful tool for the solution of such problems. The use of the melting point and boiling point as a test of purity is based on the theory of the phase rule and has been of the greatest help in organic chemistry, but in the case of many substances such as the proteins, these properties cannot be used and it is necessary to make use of an entirely analogous property, the solubility. Thus Landsteiner and Heidelberger (1) were able to show that hemoglobins of closely related animals could be distinguished by solubility measurements, and Sørensen (2) has found that proteins which had been considered as pure compounds for years were, in reality, mixtures or solid solutions of several proteins.

There is an extensive literature (3) on the theory of the solubility of both mixtures and solid solutions but practically all the work has been in relation to the study of mixtures or solid solutions of known components. The problem has been to define the property of the mixture or solid solutions from the properties of the components. In the case of an unknown material, however, the problem is to define the

properties of the components from the solubility of the mixture and to determine to what extent differences in the solubility, or in the relative amounts of the components, will affect the total solubility or the ratio of the components in the various phases. Without this information it is not possible to say definitely what possible systems may be represented by a given series of experimental results and what may not.

The following discussion is restricted to two components.

The behavior of a system consisting of two solid and one liquid phases may be completely predicted by means of the ordinary phase rule diagram. The phase rule, however, furnishes very little information as to the behavior of a solid solution in contact with a liquid. In this case there are three components and only two phases (assuming no vapor present), so that the system has three degrees of freedom even when the solid phase is present. In order to predict the solubility of a solid solution therefore, or to interpret the curves obtained, it is necessary to assume some relation between the composition of the solid phase and the solubility. The problem, as Van't Hoff and Nernst pointed out (4), is analogous to that of the vapor pressure of a mixture of two miscible liquids, the osmotic pressure in the case of the solution being analogous to the vapor pressure in the case of the liquids. Raoult's law, which states that the vapor pressure of one component of a mixture of liquids is proportional to the mole fraction of that component in the liquid mixture times the vapor pressure of the pure liquid, is the most general theoretical relation between the vapor pressure and composition of liquid solutions. The law holds accurately only in rare cases in which the liquids are closely related, but is a valuable guide in most cases. It will be assumed in the following discussion that the systems obey the phase rule and also Raoult's law. The evidence in favor of the validity of these relations as applied to solid solutions, and especially to proteins, may be summarized.

The Phase Rule.—The question has frequently been raised as to whether the phase rule applied to colloidal solutions in general and to proteins in particular. If the system is in equilibrium the phase rule must be applicable, but it is quite possible that other variables such as the surface energy or diameter of the particles must be considered as well as the usual temperature, pressure, and concentration. If this

were true the rule would be of little practical value. The work of Sørensen (2) and of Cohn (5) has shown, as a matter of fact, that proteins behave just as other compounds and that the solubility curves are those predicted for such systems by the phase rule. The results obtained with pepsin (6) also agree with the phase rule. Since the phase rule applies only to systems at equilibrium it is necessary to be sure that the values obtained are equilibrium values. In the case of solubility relations this can best be done by approaching equilibrium from the supersaturated and also from the undersaturated side. If these two values agree they must represent an equilibrium value. A second point of great importance is the choice of components. In the case of proteins dissolved in salt solution, for instance, the entire salt solution, no matter what its composition, may be considered as one component provided the composition of the salt solution is always the same, *i.e.*, the precipitate contains no salt nor water. Under these conditions a solid protein in equilibrium with a salt solution consists of two phases and two components and has therefore two degrees of freedom, so that if the temperature and pressure are fixed the concentration of the solution is fixed and cannot be varied by varying the quantity of either phase. If, however, the solid phase contains water or some of the ions or salts present in solution, as well as the protein, then the system has three components since three values, water, salt, and protein, are needed to express the composition of the phases; and the system has three degrees of freedom even in the presence of the solid phase. That is, the concentration will vary with the amount of solid present as well as with the temperature and pressure. Sørensen has found that the neglect of this possibility was the cause of the apparently abnormal results obtained by earlier workers in connection with the solubility of egg albumin.

Solid Solutions and Raoult's Law.—Raoult's law predicts that the solubility (vapor pressure) of a mixture of two miscible liquids (or solids) will be represented by a line connecting the vapor pressure (solubility) of the two pure components. The study of isomorphous mixtures of isomers has shown that the solubility of such mixtures may sometimes be predicted from Raoult's law, as in the case of the camphor oximes (7); but that in other cases the solubility of such mixtures may show a maximum or minimum, which is contrary to

Raoult's law. Landsteiner and Heidelberger (1) have found that mixtures of hemoglobins from two closely related animals have solubilities between those of the two separate proteins, so that they form solid solutions and agree qualitatively at least with Raoult's law. Sørensen's (2) results also indicate that solid solutions are of common occurrence with proteins, so that it is a possibility which must always be considered. There is, however, no reason to suppose that Raoult's law holds in general in case of solid solutions and predictions made from it must be considered only as approximations.

Solubility Curves of Mixtures from the Phase Rule

The solubility of mixtures of various proportions of two substances in a liquid may be represented conveniently on the ordinary phase rule

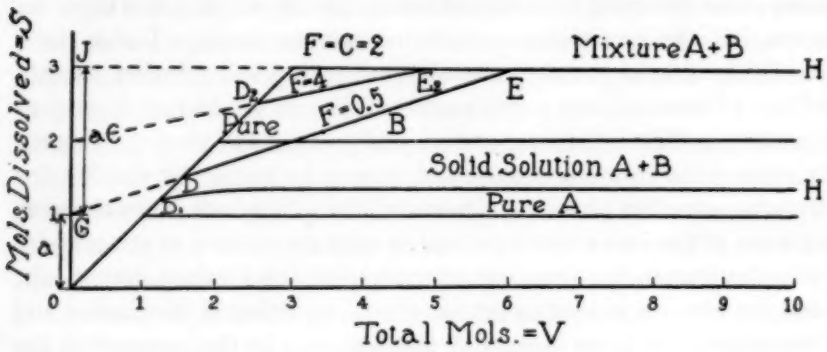

FIG. 1

diagram when the composition of the mixtures is known. In the case of an unknown mixture the problem is to derive the solubilities and relative amounts of the components of the mixture from the solubility curve of the mixture when added in increasing amount to the liquid. This can be done if it be further assumed that the mixtures form ideal solutions, *i.e.*, that the solubility of one is independent of the presence of the other. Assume that a solid material consists of a mixture of A moles of A and FA moles of B. The ratio of B to A is then F. The solubility of A is a and of B is Ca, A is the less soluble so C is greater than 1. Increasing amounts of the mixture are added to unit volume of liquid and the total amount of solid in the solution determined.

This amount is plotted as ordinate against the total amount of the mixture added as abscissa (Fig. 1). At first all the solid added will dissolve and the points will fall on a line with a slope of 45° (0-*D*). As soon as an amount of *A* or *B* equal to its solubility is added, solid will appear and there will be a break in the curve as at *D*. The system has now two phases and three components and the concentration can still change with the amount of the phases present. As more solid is added the solubility will continue along the line *D*-*E*, until at *E* the second solid phase appears. There are now three phases and three components, so if the temperature and pressure are fixed the composition is fixed and there will be no further change in concentration with added solid. It is now possible to determine *F*, *C* and *a*, either graphically or algebraically.* *D*-*E* and *E*-*H* are continued back to the solubility axis. The distance $0G$ is therefore *a* or *Ca* and *GJ* is either *Ca* or *a*. Since *C*, by definition, is more than one the larger of these values is *Ca* and the smaller is *a*. The slope of the line *D*-*E* is $\frac{F}{1+F}$ if $0G = a$ and $\frac{1}{1+F}$ if $0G = Ca$. When $F = C$ both solids appear together so that the solubility curve shown in Fig. 1 for $F = C$ has the same form as for a pure substance. If $F > C$, *B* (the more soluble) will appear first as solid while if $F < C$, *A* (the less soluble) will appear first. The diagram shows also that in the part of the curve between *D* and *E* the solid phase will be either pure *A* or pure *B* and that the extent of the range over which the pure solid is present depends on the difference between *C* and *F* and is zero when $C = F$. Evidently then this is the most significant part of the curve for deciding whether or not a given material is a mixture and analysis of the first precipitate to appear is the most sensitive test. If this agrees with that of the original material, or if its solubility is the same as that of the original material, then either there is very little of a second substance present or the solubilities of the two are very nearly in proportion to their relative amounts. In case it were suspected that one property of a material were due to one substance and another property to another substance the ratio of the two properties in the first precipitate to appear would serve as a very sensitive test, since at that point the ratio of *A* to *B* must differ greatly from that of the original mixture even though the amount of *B*

* See equations on pages 787 and 788.

is so small as not to affect the shape of the total solubility curve and the range of existence of pure A or B so small as to render it impossible to actually isolate pure A or B. Still, near this range the ratio of A to B would be different from that in the original mixture. If the ratio of A to B may be measured therefore the possibility of a small per cent mixture may be ruled out and the material must be either a pure substance or the ratio of the solubilities of the two components must be very nearly identical with their relative amounts. If in addition, this were found to be the case in several solvents it would be practically certain that the material was a pure substance since it is highly improbable that the relative solubilities of two substances (except allotropes or polymorphs) should be identical in various solvents.

Solid Solutions

The question as to whether a given material is a mixture may therefore be settled with practical certainty by solubility measurements in various solvents. Unfortunately, however, in the case of solid solutions the conclusions are much less definite. All that can be learned from the phase rule is the point at which the solid will appear if the relation connecting the solubility of the solid solution in terms of the solubility of the pure components and of the percentage composition is known. Since in this case there is only one solid phase the system will still have three degrees of freedom after the solid appears and the concentration in the solution will vary with the amount of solid phase. No information as to the nature or extent of this variation can be gained from the phase rule alone. If, however, the system be assumed to follow Raoult's law and the relative proportions and solubilities of the two components are known, then the form of the solubility curve and the ratio of A to B at any point can be calculated. Conversely, if the solubility curve is known the relative solubilities can be calculated. In order to define the system, however, it is necessary to know three points on the solubility curve in the case of a solid solution instead of two as in the case of a mixture.

Form of the Solubility Curve.—The curve for the total solubility resembles that for the case of a mixture but has only one sharp break at D. From D on the curve rises, the slope becomes less as more and more mixture is present, and approaches zero asymptotically. The

curve will lie between that for pure A and pure B and will approach the curve for either one or the other as F varies from zero to infinity. If $C = 1$ the curve will have the form of that for a pure substance and the total solubility will be independent of the amount of the solid present. In this case the ratio of A/B would be the same at all points and there would be no indication of the presence of more than one substance. If C has any value but one, however, the ratio of A/B in the first precipitate that appears will be $1/FC$ while that of the original solid solution is $1/F$. As the amount of solid present increases the ratio of A/B in the solid approaches $1/F$ while the ratio of A/B in solution approaches $1/FC$. At all points the ratio of A/B in solution to A/B in the solid equals $\frac{1}{C}$. As in the case of the mixture therefore the best test for the presence of two hypothetical components is the ratio of the two properties in the solid, when the material is nearly all dissolved, as compared with the ratio in the solution when a large amount of solid is present.

These conclusions may be obtained algebraically from Raoult's law as outlined below.

Mixtures

A the less soluble

a = solubility A

A = moles A = $\dfrac{V}{1+F}$

Ca = " B

$(C > 1)$

FA = moles B = $\dfrac{VF}{1+F}$

Total moles dissolved = S

Total moles in system = $A + FA = V$

Total volume = 1

Then

From $0 - D$

$$V = S \tag{1}$$

From $D - E$

$$S = a + \frac{VF}{1 + F}, \text{ if (Intercept) } 0\,G < G\,J \tag{2}$$

or

$$S = Ca + \frac{V}{1 + F}, \text{ if (Intercept) } 0\,G > G\,J \tag{3}$$

From $E - H$, $S = a + Ca$

or at D, $S_D = V_D$

$$F = \frac{S_D}{a} - 1$$

or

$$F = \frac{Ca}{S_D - Ca}$$

From (2) or (3)

When $V = 0$

$$S = a \text{ or } Ca = 0\,G \tag{4}$$

Conditions determining which solid appears first.

V_A = value of V when A appears

V_B = " " " " B "

Then $\frac{V_A}{1 + F} = a$ $\qquad$ $\frac{V_B F}{1 + F} = Ca$

$$V_A = a\,(1 + F) \tag{5}$$

$$V_B = \frac{Ca}{F}\,(1 + F) \tag{6}$$

Divide (6) by (5)

$$\frac{V_B}{V_A} = \frac{C}{F}$$

$\therefore$ if $F < C$, $V_B > V_A$ and A appears first

" $F = C$, $V_B = V_A$ " both appear together

" $F > C$, $V_B < V_A$ " B appears first

Solid Solutions

x = moles A in solution

y = " B " "

$x + y = S$

Raoult's law

$$x = \frac{\left(\frac{V}{1+F} - x\right) a}{\left(\frac{V}{1+F} - x\right) + \left(\frac{VF}{1+F} - y\right)} \tag{1}$$

$$x = \frac{\frac{a\,V}{1+F} - a\,x}{V - S} \tag{1 a}$$

$$y = \frac{\left(\frac{VF}{1+F} - y\right) Ca}{\left(\frac{VF}{1+F} - x\right) + \left(\frac{VF}{1+F} - y\right)} \tag{2}$$

$$y = \frac{\frac{Ca\,VF}{1+F} - Ca\,y}{V - S} \tag{2 a}$$

$$x = \frac{a\,V}{(1+F)} \cdot \frac{1}{(V - S + a)} \tag{3}$$

$$y = \frac{a\,V}{1+F} \cdot \frac{C\,F}{V - S + Ca} \tag{4}$$

$$S = x + y = \frac{a\,V}{1+F}\left(\frac{CF}{(V-S+Ca)} + \frac{1}{(V-S+a)}\right) \tag{5}$$

$$\frac{x}{y} = \frac{1}{F\,C} \cdot \frac{V-S+Ca}{V-S+a} \tag{6}$$

From (5) a, F and C may be solved numerically from 3 points on the solubility curve.

When $V - S \doteq 0$ (very little solid)

$$S_D = \frac{a\,C\,(1+F)}{C+F}\,*$$

and when large excess solid ($V >> S$)

$$S_H = \frac{a\,(1+F\,C)}{1+F}$$

also, if $C = 1$

$$S = a$$

From (6) $V - S \doteq 0$

$$\frac{x_D}{y_D} \doteq \frac{1}{F}$$

---

* This relation is derived as follows:

Let $V - S = U$; also let S_D be the value of S when U approaches zero; *i.e.*, $S_D \doteq V_D$

From (5) $U = S_D\left[1 - \frac{a}{1+F}\left(\frac{CF}{U+Ca} + \frac{1}{U+a}\right)\right]$

$$\text{or } S_D = \frac{U}{1 - \frac{a}{1+F}\left(\frac{CF}{U+Ca} + \frac{1}{U+a}\right)} \doteq \frac{0}{0}$$

The value of S_D is found by differentiating the indeterminate fraction with respect to U and finding its limiting value as U approaches zero.

and if $V - S \doteq V$ (large excess solid)

$$\frac{x_H}{y_H} \doteq \frac{1}{F\,C}$$

also if $w = A$ in solid $= \dfrac{V}{1 + F} - x$

$$z = B \text{ in solid} = \frac{VF}{1 + F} - y$$

Substitute in (1) and (2)

$$x = \frac{w\,a}{w + z}$$

$$y = \frac{Ca\,z}{w + z}$$

$$\frac{\frac{x}{y}}{\frac{w}{z}} = \frac{1}{C}$$

SUMMARY

It is shown that the relative amounts and the solubilities of the two components of a mixture or solid solution may be calculated from the solubility curve of the mixture or the solid solution.

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THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

I. Intact Valonia ventricosa

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Many living systems adhere to Ohm's law unless the applied voltage exceeds a certain limit above which the apparent resistance to direct current does not remain constant but decreases (Strohl[1]). This is one of the many resemblances to electrode phenomena which have been pointed out by Gildemeister.[2] Probably all living tissues have a lowered resistance as one of the aspects of injury by electrocution.[3]

An apparent *increase* of resistance, as measured in the Wheatstone bridge during the application of increased direct potentials, has not often been observed, but occurs in several of the large plant cells studied in this laboratory. For example, the resistance of *Nitella*, ordinarily very high in most solutions and constant under 5 to 100 mv. applied potential (unless stimulation occurs[4]) may be lowered by contact with KCl; after this the application of relatively high voltages increases the resistance at the point where the current passes from the external solution into the protoplasm. *Valonia macrophysa*, measured very soon after its collection in Bermuda, may also have a resistance which varies with the applied potential, and is then usually greater when the current passes in one direction than in the other. This seems

[1] Strohl, A., La conductibilité electrique du corps humain, Paris, 1925.

[2] Gildemeister, M., *Handb. norm. u. path. Physiol.*, 1928, **8**, pt. 2, 657.

[3] Injury and recovery after the application of high voltages to plant tissue have recently been studied by Dixon and Bennet-Clark, who also found a small *rise* of resistance to follow after very short stimuli of certain voltages (low frequency alternating current). *Cf.* Dixon, H. H., and Bennet-Clark, T. A., *Proc. Roy. Dublin Soc.*, 1927, **18**, 351; 1928, **19**, 27.

[4] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 495

to be due to injury at one end, where the attachment to a neighboring cell has been broken. *V. macrophysa*, of Tortugas, seldom entirely recovers from the injuries of separation and cleaning and its resistance remains low and variable during its life in the laboratory (which, in the summer, is usually cut short by zoosporulation).

The variation of resistance with potential is most characteristically shown in the freshly gathered cells of *Valonia ventricosa*. Preliminary notes[5] have indicated the character of this effect, which is here more completely described. Kept in the laboratory, the cells reach a condition in which their resistance is high and does not change either with time or with variations in voltage.[6] We shall call this the "constant state." But for a long period, while this state is being approached, the resistance is low if low potentials are applied and rises when the potential is increased. The amount of the rise is largest at first and becomes less as the cells approach constancy. Some of the smaller cells never show the variability; others which have nearly lost it may be made to display it again in larger degree simply by repeated measurement. Impaled cells show the effect most strikingly[7] and contribute largely to the proposed explanation. Their behavior will be further discussed in another paper.

The experiments here reported were performed with *intact* cells, measured soon after collection at the Dry Tortugas, Florida, in the months of June, July, and August. The temperature of the air, and of the sea water in which the cells lived, was between 25° and 30°C. The methods and apparatus employed in the study of the variable state are the same as used to measure protoplasmic resistance in the constant state, as previously described.[6]

The electrical circuit was an equal ratio-arm Wheatstone bridge with non-polarizable electrodes. In some cases, measurements were made with the string galvanometer connected directly into the bridge without the vacuum tube detector. This was possible on account of the low resistances of *Valonia* cells. The values were read directly from the decade boxes at balance, or, in the case of resistances

[5] Blinks, L. R., Carnegie Institution of Washington Year Book No. 26, 1926–27, 217; No. 27, 1927–28, 270. *Am. J. Physiol.*, 1928, **85**, 351.

[6] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

[7] Blinks, L. R., Carnegie Institution of Washington Year Book No. 28, 1929, 277.

changing rapidly during current flow, by photographically recording the moving string image. Calibration was automatically obtained at any given value of resistance and potential, by the known increments of resistance (in 100 or 1000 ohm units) which were made to keep the image upon the photographic paper.

The applied potential was read directly on a millivoltmeter (a Weston milliammeter adapted by series resistance to register one-half the potential drop through the entire bridge). This gave essentially the potential drop across the cell at balance. It was frequently calibrated against a potentiometer, and was easily read to about 5 per cent.

The electrodes were carefully prepared to avoid appreciable polarization at any of the current densities employed. Large sheets of lead, amalgamated with mercury and coated electrolytically with chloride, were dipped into cups filled with acidified sea water. These cups were connected to a second pair of vessels bearing the cell holders, by large blocks of agar imbibed with sea water. These were frequently renewed to avoid diffusion from the electrode vessels.

The cells were supported in circular holes cut in stiff agar blocks which maintained a slight pressure on the ends of the cells. These blocks were imbibed, and the wells filled, with the desired solution (usually sea water) through which the electric current passed in entering and leaving the cell. Leaks of fluid were prevented by covering the tops of the wells tightly with glass or agar slips, the residual seepage being carried away by filter paper drains laid close to the contacts. Under these conditions the excess moisture drained off the cell and its resistance became stationary in 3 or 4 minutes (aside from inherent variations in the cells).

It should be emphasized that neither dead cells, nor air-filled walls, nor any simple electrolytic conductor, showed the effects to be described. Large currents were sent through these for long periods of time without appreciable change of resistance. The variation of resistance with potential was only found in the living cells. Unless otherwise noted, the resistance values of the latter are those attained in the "steady state" after the "transient effects" are over, and no further change in the current is occurring. They thus include any polarization potentials in addition to true ohmic resistance.

A resistance that apparently increases with potential must have a lowered initial value, and this is characteristic of the freshly gathered cells of *V. ventricosa*. Their resistance is often only a third or a half that attained when the cells have reached the constant state. Since the resistance of the cell wall, as a shunt around the protoplasm, remains nearly the same during this time, the changes in the protoplasm itself are relatively still greater.

As an example: a freshly gathered cell, 2 cm. in diameter, and free

of adhering growths, had a resistance, when placed in the apparatus, of 700 ohms, rising slowly to 800 ohms, when measured under an applied potential of 20 mv. The resistance rose to 1300 ohms when the potential was increased to 75 mv., and fell off again at potentials above this. It returned to 750 ohms at 20 mv. A cell of similar dimensions, which had stood quietly in sea water in the laboratory for 4 weeks, had a resistance between 2100 and 2200 ohms; this only increased about 5 per cent over the range of 15 to 100 mv. applied potential.

When the first cell was killed its resistance dropped to 20 ohms at all potentials. Its sap and protoplasm were then removed, the cellulose wall was well washed out with sea water, and inflated with air. This wall had an average resistance of 2450 ohms (at all potentials), when measured in the same position as the cell, and under the conditions detailed in the previous paper.[6] This value makes it possible to calculate what the *protoplasmic* resistance was in life. By the method of calculation previously described[6] it was found to have been only 920 ohms when measured at 20 mv., and to have risen to 2770 ohms at 75 mv. Thus an increase of 200 per cent in the living protoplasmic resistance had occurred under the influence of the increased voltage.

Cells showing a smaller variation in resistance have higher initial values for the protoplasm. The isolated wall of the nearly constant cell used for comparison above, had an average resistance of 2380 ohms. Its living protoplasmic resistance was therefore between 18,000 and 29,000 ohms. This approaches the values found in the constant condition of the cells, in which the resistance of the living protoplasm may be 50,000 ohms or more.[6]

The cells usually tend to rise in resistance after collection, but the increase is not always gradual and there are often variations from day to day, apparently brought about by minor mechanical injury. The extreme sensitivity of the cells to handling at this stage is shown by the following example:

A cell was measured immediately after collection, without cleaning or drying. The resistance rose from 1000 to 1200 ohms during 2 minutes of measurement at 25 mv. Ten minutes later, however, the resistance had fallen to 700 ohms at 25 mv. A brief application of 100

mv. brought it back to 1200 ohms, but it fell to 680 ohms at 25 mv. again. The cell was then removed and wiped very gently on a towel for only 30 seconds. On replacing it, the resistance had fallen to 140 ohms, and in 1 minute more was only 60 ohms. Removal to sea water failed to cause recovery and 1 hour later the dead resistance was 40 ohms. The average resistance of the air-filled wall was 1650 ohms, giving for the live protoplasm a maximum resistance of 4400 ohms, which decreased nearly to zero during the experiment.

The cells at this stage are often injured by continued measurement or long sojourn in the apparatus. Frequently a cell which has stood several days without disturbance in sea water has a very high resistance when first placed between the agar blocks. One cell reached in 3 minutes a resistance, measured at 25 mv., of 3800 ohms, which was slightly increased by application of 100 mv. (to 4000 ohms). The resistance soon began to drop, however, and in 8 minutes had reached 1250 ohms (at all potentials). At 900 ohms it was removed to sea water for recovery. The next day it appeared well, and gave an initial resistance of 3000 ohms at 25 mv. But the value soon decreased, and in 6 minutes was 2000 ohms with the current in one direction, 1800 ohms in the other. 100 mv. caused a rise to 3300, and to 2900 ohms, respectively, and during a long application the resistance rose to 3700 ohms. When the cell was killed 4 hours later the average resistance of the wall was found to be 4100 ohms. This agrees fairly closely with the values of the first measurement (and less closely with the highest later attained under 100 mv. applications).

When we calculate what the protoplasmic resistance had been in the living state, we find that it was between 52,000 ohms and 166,000 ohms at first, but that it fell to 1150 ohms during the measurement. On the second day it had recovered to 11,000 ohms at 25 mv. potential, and the application of 100 mv. raised it to 38,000 ohms. Very large fluctuations had thus occurred during life.

The values of resistance attained under the application of high potentials are shown in Table I, for a group of variable cells. It is seen that the higher resistances reached are from 50 to 90 per cent of the value for the isolated cell wall, and that the values for low potential are in general about half that for high potential. (The differences, of course, fall off as the cells approach the constant state of resistance.)

The ratio of low to high values suggests that the two levels of resistance correspond to two current paths of nearly equal resistance, both of which conduct at low potentials, but one of which becomes non-conducting at higher potentials. There are two circuits in which this is possible. These are shown in Figs. 1 and 2.

In the case of Fig. 1, we assume that the current does not pass across the protoplasm into the vacuole, but that under low potentials it

TABLE I

Apparent Direct Current Resistance Levels of Variable Valonia ventricosa

Cell No.	Living cells				Dead cells	
	Low value		High value		Resistance*	
	Potential	Resistance	Potential	Resistance	Dead cell	Isolated wall filled with air
	mv.	*ohms*	*mv.*	*ohms*	*ohms*	*ohms*
4	25	2000	100	4600	55	4500
23	22	1200	100	2000	—	—
25	25	1000	100	2100	—	—
27	20	1500	100	2800	38	4200
30	25	1450	100	2350	—	—
32	25	1000	100	1700	27	2500
34	25	3600	50	4300	53	4700
44	25	1280	100	1570	40	2000
45	25	1600	100	2300	40	2100
51	25	2200	75	3800	50	4000
54	25	1600	75	2100	—	3300
55	25	1400	100	1690	—	1700
59	25	830	100	910	—	1200
60 (1st day)	25	1900	150	2700	—	—
" (2nd day)	25	3600	75	3800	63	4150

* These resistances do not vary with the applied potential.

may pass around the cell through the wall, and also through the protoplasm just beneath the wall. Since the protoplasm is of about the same thickness as the wall, the relation of resistance levels would be readily explained by the assumption that it consisted of 3 layers (*W* being imbibed with sea water), and that the outer layer *X* admitted current at low potentials, but became highly resistant at higher values (*Y* remaining a poor conductor at all times). The cur-

rent would thus find two nearly equal paths at low potentials but only one (the wall) at higher potentials.

This assignment of the current paths is attractive in that it explains how the cells may have a high resistance and still maintain a cell sap of high potassium content by the impermeability of *Y*. It is also partially favored by the failure to detect an increase of sulfate ion in the vacuolar sap even after current under low potential had been passed through cells for 1 hour or more from sulfate sea water[8] (the

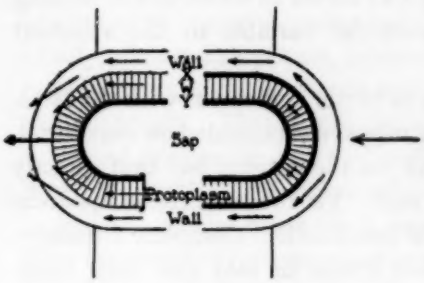

FIG. 1

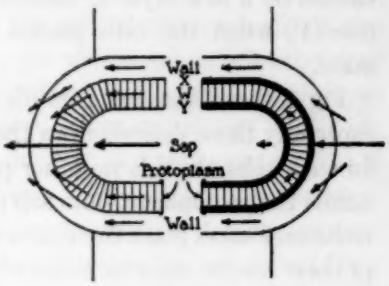

FIG. 2

FIG. 1. Diagram of suggested current paths in the variable cell (the length of the arrows indicates the magnitude of the current). At the left the current is shown equally divided between the wall and the protoplasm. This represents the low resistance value. At higher potentials the condition at the right may be produced, in which the layer *X* has become almost non-conducting so that almost all of the current is confined to the cell wall. In either case, little or no current passes into the sap. (The highly resistant layers are indicated by heavy black lines.)

FIG. 2. Diagram of current paths which represents the apparent condition in variable cells (the length of the arrows indicates the magnitude of the current). At the left much of the current is shown passing across the protoplasm from sap to contact. At higher potentials the condition shown at the right is produced, in which the resistance of the protoplasm has greatly increased, confining most of the current to the cell wall. (The highly resistant layers are indicated by heavy black lines.)

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[8] This had the following composition:

Na_2SO_4	0.4 M	1000 parts
K_2SO_4	0.4 M	20 parts
$MgSO_4$	0.6 M	116 parts
$CaSO_4$	(saturated)	

resistance remaining at the low level throughout). But calculation shows that the migration of any ion would be extremely small over this period at the necessarily small current density employed. The resistance rises in larger currents and a much longer exposure in the apparatus is usually injurious to the cells. On the other hand the less certain criteria of alternating current measurements show that the differences between the low and the high levels of resistance are not paralleled by the expected reduction of capacitance which would be caused by a new layer of dielectric (X) added in series to the existing one (Y) when the cells passed from the variable to the constant state.

Fig. 2 shows assumed conditions in better agreement with the facts, especially those derived from the study of cells impaled on capillaries. In such cells there is no other path for the current but that directly across the protoplasm, from sap to wall. Yet the same rise of apparent resistance takes place there as in the intact cells. Complete discussion of these results must be deferred, but it may be said that their essential features are duplicated in the experiments performed on intact cells which have been chloroformed at one end. These give virtually a contact with the sap at the killed end, and the current passes across but one layer of living protoplasm.

The tightly fitting contact of the cell with the agar makes it possible to apply chloroform at one end so that it cannot reach the other end except by diffusion through the sap. This is so slow that for a few minutes after the chloroformed end is completely dead the other end is nearly normal. In performing the experiment sea water saturated with chloroform is substituted for the sea water in the well, or a small drop of chloroform may be introduced into the well, which is then covered again.

The prompt effect of the chloroform is to cause a deflection of the bridge detector, corresponding to a potential difference in the cell up to 20 or 25 mv., the killed end being positive. (This is in agreement with the impaled cells of *V. ventricosa*, in which the inside (sap) attains a value of 15 to 25 mv. positive.[7]) This potential difference is balanced out by a series potentiometer, or the deflected state may be used as a pseudo-zero.

When the variable cells are thus chloroformed at one end quite different resistances are found, depending on the direction in which the current flows through the cell. These become most marked at the higher potentials. Below 20 mv. the resistance is somewhat less

than in the unchloroformed cell and remains about the same with the current passing in either direction. But as the positive current is increased *outward* across the living end, the resistance begins to *decrease* with increasing potential, and may be nearly halved at 100 mv. Only when the positive current is passed *inward* across the uninjured protoplasm does the resistance *rise* with potential.

The variable and directional effects are most marked for about 10 or 15 minutes after chloroforming and then begin to fall off as the total resistance decreases. This is taken as meaning that the chloroform has begun to injure the other end of the cell, which eventually is also killed. Before this occurs, however, the effects are sufficiently clear to explain the essential phenomena of current flow across a single layer of protoplasm. The results are shown diagrammatically in Fig. 3. *A* is the resistance curve of cells in the constant state. For a range of over 100 mv. either side of zero, the resistance is constant. It falls off as the breakdown value is exceeded, but more rapidly when the positive current passes *outward* (from sap to sea water) than when it passes *inward*. Curve *B* represents the variation of resistance in a cell which has nearly recovered constancy. The maximum rise of resistance between zero and 100 mv. with inward current is only about 5 per cent, but the resistance falls rapidly when the current passes outward. Curve *C* is that of the typical variable cell, in which with inward current the resistance rises over 100 per cent with the increase of potential between zero and 100 mv. An outward current causes a smaller decrease of resistance. Curve *D*, which represents an impaled cell, is included to show the extreme of the variable condition, in which the resistance is nearly at zero with small currents in either direction, but increases greatly with larger inward currents. Increased outward currents, on the contrary, have very little effect, since the resistance is already very low. Curve *E* shows that in dead cells the variations are absent, and the resistance is low at all potentials.

The shape of these curves is rather variable, and depends in large degree on the immediately preceding history of the cell. If a current has been passed shortly before, the whole curve may be shifted to the right or left within the limits represented by *B* and *D*, depending on the direction in which the current has passed (an explanation of this will be suggested presently).

All the levels mentioned are those of the steady state in which the resistance has reached a value that no longer changes with time during the current flow. But the speed with which this steady state is attained is highly dependent on the previous treatment, and varies with the length of time between applications of potential. Nothing has previously been said of the time course of these resistance changes,

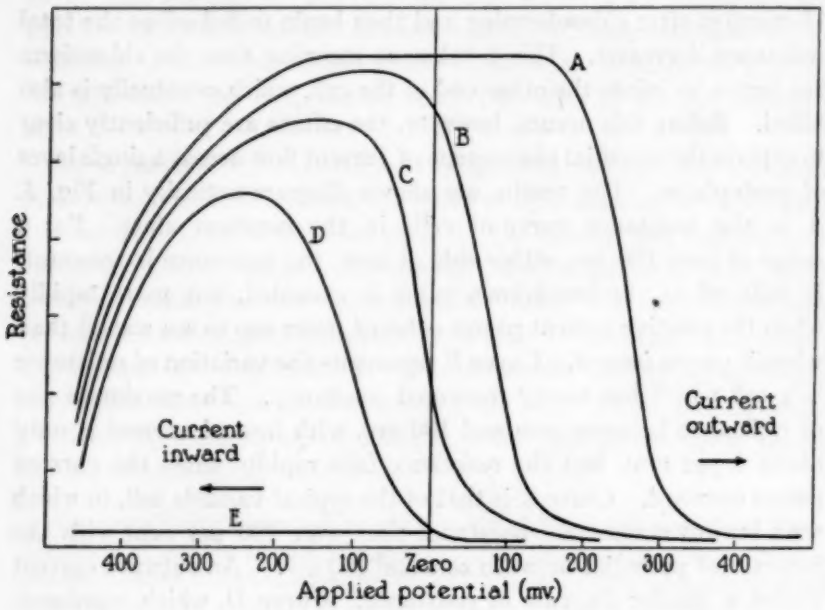

FIG. 3. Diagrammatic graph showing the variation of resistance with potential across a single layer of *Valonia* protoplasm. The resistance is in arbitrary units, the applied potential is in millivolts, increasing on either side of zero; to the left there is an increasing positive current from sea water to sap; to the right, from sap to sea water. Curve *A* represents the constant condition, *B* and *C* the variable condition of intact cells, *D* that of an impaled cell, and *E* that of a dead cell.

and they are more properly reserved for discussion in another paper with the "transient effects" which occur at every application and removal of potential across the cells. They are, however, of value in analyzing the cause of the resistance rise.[9]

[9] Blinks, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 359. An expansion of this preliminary note is shortly to be published.

A very small current may pass through the cells for a long time—up to an hour or more at 20 mv.—without effect either on the resistance or the back E.M.F. A slight increase of potential above this is also

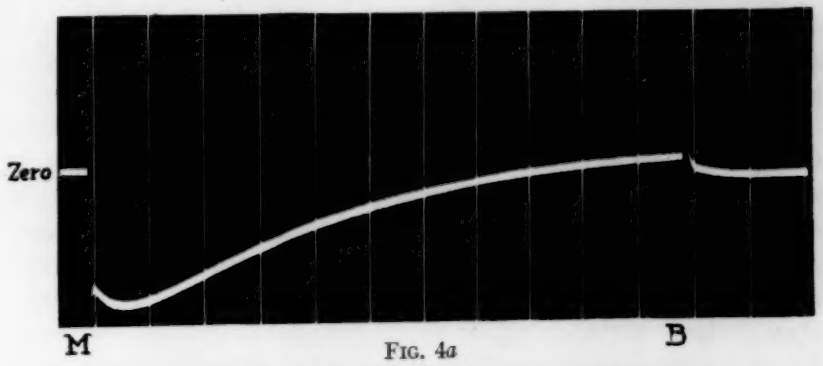

FIG. 4*a*

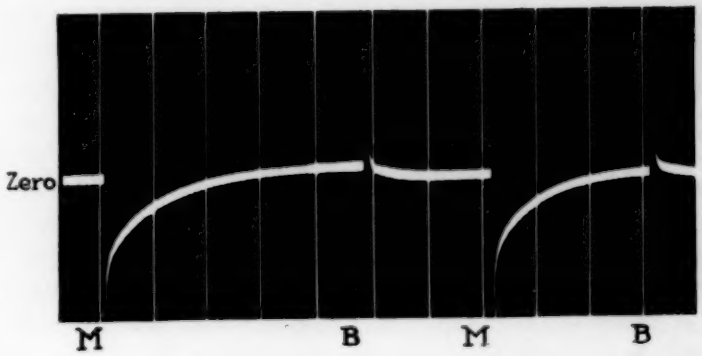

FIG. 4*b*

FIG. 4*a*. String galvanometer record of the resistance rise under the application of 75 mv. to a variable cell. The bridge has previously been balanced to the steady state value so that the curve rises to zero. *M*, make, *B*, break, of voltage applied to bridge. Time marks, 1 second.

FIG. 4*b*. (Continuation of 4*a*.) String galvanometer record of second and third applications of 75 mv. in immediate succession after 4*a*. Details as in 4*a*.

ineffective at first. But as the current flow continues the galvanometer deflects, at first slowly, then more rapidly, and finally more slowly again, to reach what resembles a steady state. If the measuring potential is now removed, a much larger back E.M.F. is discovered,

and a current flows for a moment in the opposite direction, the zero line being approached by a regular curve.

The application of the same potential again causes a regular rise of apparent resistance, but this time more rapidly, showing that an effect of the previous exposure persisted. A third application in quick succession is usually still faster. Fig. 4 shows a typical string galvanometer record of such a series of applications.

Several facts come out of the study of such records. It is evident that part or all of the apparent rise of resistance is due to a back E.M.F. developed by the current. This has the effect of a capacity (either polarization or static) but it differs from these in that the time constant is quite different for the charge and the discharge, the first charge taking 10 to 20 times as long as the discharge. The time constant of charging also changes from one exposure to the next, becoming less with succeeding applications. Even after several exposures have been made, the curves do not reach that equality and symmetry of rise and fall which characterize condenser and electrode charge and discharge (the "superposition law" of Curie[10]). Since cells in the constant condition have very symmetrical charge and discharge curves (*cf.* the curves for *Nitella*[4]), it seems probable that another process is occurring in the first part of the resistance rise in the variable cells. This in effect is a change of the protoplasm from an electrode fairly reversible to the ions passing, to an electrode nearly or entirely irreversible to them, and finally becoming polarized with an opposite and nearly equal E.M.F. The question occurs whether it is the nature of the protoplasmic electrode that is being changed (as if by a decrease in pore size) or whether the kinds of ions in the current are changing. The latter is the simpler explanation, and has considerable evidence in its favor. The mechanism for this effect is suggested by the thin collodion membranes discussed in another paper.[9] In these the resistance is found to be dependent on the applied potential, rising when sodium ions are carried across it, and falling when potassium ions are so carried. In such collodion the resistance rise is real, due to the different ion mobilities, and not to a back E.M.F. of polarization. But the mechanism is suggestive of what might

[10] Curie, J., *Ann. chim. et phys.*, 1889 (6), **18**, 203.

happen in a membrane which allowed one kind of ion to pass freely, while polarizing when presented with another kind.[11] Small currents would not carry away the first kind any faster than they diffused back, but greater currents would decrease their concentration, and polarization would then occur. In so-called "non-polarizable" electrodes an apparent increase of resistance may be brought about by large current densities in this manner.

The question remains, what are the ions which the protoplasm allows to pass in its variable state? The high concentration of KCl in the sap, and the fact that the resistance falls and polarization decreases, when the current passes outward from the sap across the protoplasm suggests that it is potassium ions which have a high mobility in the variable *Valonia* cells. There is considerable evidence that potassium remains trapped for a long time in the protoplasm, and its presence is apparently responsible for the shift of the curves in Fig. 3 toward *D*. As the cells recover, this potassium is lost by diffusion or by absorption into the vacuole, and the highly resistant level of *A* is reached. However, application of 0.6 M KCl to the outside of the cells produces very little effect for considerable time, and there is even the anomaly, during part of the exposure, of a shorter time constant of polarization in KCl than in sea water. But it should be said that great difficulty is met in interpreting the effects of single salts, since they produce alterations in the protoplasm. A large amount of KCl in sea water sometimes has an effect in preventing the rise of resistance in the variable state, but this disappears as the cells approach the constant state. Further evidence on the reversibility to ions will be given for impaled cells in another paper.

The ultimate cause of the variable state has been ascribed to mechanical injury,[6] but the other alternative might be to regard the variable state as the natural, growing condition of the cells, as opposed to an artificial, highly impermeable state which they may reach in the laboratory. However, many cells have a constant high resistance from the beginning, and can later be made variable by mechanical injury such as impalement.[7] Reference has been made in a preceding paper[6] to the ease with which *V. ventricosa* protoplasm breaks up into

[11] *Cf.* Labes, R., *Arch. exp. Path. u. Pharm.*, 1927, **125**, 29; Zain, H., *Ibid.* 53; Labes, R., and Zain, H., *Ibid.*, **126**, 284, 352.

hundreds of tiny spheres which are new cells inside the old wall. It is easy to conceive this process as partly occurring, the strains in the surface greatly changing its permeability (or thickness) without gross rupture. High temperature alone (up to 30° or 31°C. at Tortugas) does not cause the variable state, since constant cells taken back from New York to Tortugas and exposed to this temperature do not develop the effect, and the recovery of variable cells takes place regularly at this temperature.

Since the variable condition is a means of producing great changes of permeability to some ions, during an apparently healthy state of the cells, it has a bearing upon injury and recovery. This will be discussed in a later paper.

Grateful acknowledgment is made to the Carnegie Institution of Washington for its generous cooperation at the Tortugas Laboratory, where these experiments were carried out.

SUMMARY

Many of the freshly gathered cells of *Valonia ventricosa* have a resistance to direct current which is variable and depends on the potential applied. It is low when low potentials are applied and rises sharply at higher values. The rise may be more than 100 per cent in the cell as a whole, which is equivalent to several hundred per cent in the protoplasm alone. The rise becomes less as the cells stand in the laboratory, until a maximum is reached at all applied potentials, low and high, below the breakdown value (about 100 mv.): the cells are then said to be in a constant state.

During the variable state, the resistance rises when the positive current enters the protoplasm from outside, and falls when it passes out from the vacuole (this is determined by killing one end with chloroform).

The rise of resistance becomes faster with closely succeeding applications of potential. This is ascribed to the removal from the protoplasm of ions to which it is reversible. There is some evidence that these may be potassium ions.

Much of the apparent resistance rise may be accounted for by a back E.M.F. caused by the flow of current.

THE ENZYMIC HYDROLYSIS OF PHLORIDZIN

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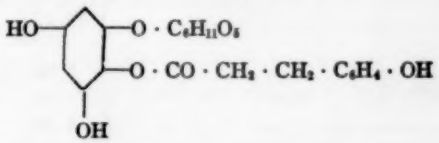
The Structure of Phloridzin

Phloridzin is the name given by de Koninck[1] to a substance which had been found by Geiger[2] to occur in the rootbark of the apple tree. The empirical formula ascribed to it by Roser[3]—$C_{21}H_{24}O_{10},2H_2O$—was endorsed by Strecker[4] after a review of the analytical data previously published by Stass,[5] by Liebig[6] and by Mulder.[7] The constitution of phloridzin[8] may be represented as follows:



Hydrolysis by dilute mineral acids yields phloretin and glucose.[9-12] Warm baryta converts phloridzin into phloretic acid and phlorin,[13] which is identical with the phloroglucinol-β-glucoside synthesized by Fischer and Strauss.[14]

The Hydrolysis of Phloridzin by Acid

A polarimetric examination of the rate of hydrolysis of phloridzin under the influence of hydrochloric acid[15] showed the reaction to be kinetically unimolecular, as for glucoside hydrolyses generally. On comparing the velocity coefficient for the hydrolysis of phloridzin with values determined for several other glucosides at the same temperature and under identical catalytic conditions,[16] it is found that phloridzin is the most labile glucoside that has hitherto been examined. Too

much stress is not, however, to be laid on the velocity constant as a guide to relative stability; the critical increment or the energy of activation for the reaction, calculated by means of the Arrhenius equation[17] is a much more significant quantity than velocity itself. Considered from this standpoint, phloridzin still appears to be the most unstable glucoside yet encountered.[18] The data quoted in Table I emphasize an analogy between phloridzin and the γ-fructosides, which is more pronounced than that between phloridzin and the normal glucosides. It should, perhaps, be pointed out that the data relating to the hydrolysis of the two trisaccharides raffinose and melezitose refer to the cleavage of the γ-fructosidic linkage in each case.

TABLE I

Compound hydrolysed	Rate of hydrolysis (seconds^{-1}) at 25°C. and pH = 0.0	Critical increment (calories/gram mole)
Maltose	6.75	30,970
Salicin	6.43 $\times 10^{-5}$	31,630
Arbutin	18.10	30,760
Phloridzin	1.96 $\times 10^{-4}$	22,920
Sucrose	1.48	25,830
Raffinose	1.23 $\times 10^{-4}$	25,340
Melezitose	0.52	25,600

The Diabetic Action of Phloridzin

The apparently anomalous behaviour of phloridzin during hydrolysis by acids is of interest in that this glucoside is exceptional also in its physiological properties. The glycosuria which van Mering[19] found was induced in animals after injection of phloridzin was shown by Moritz and Prausnitz[20] to be similar to the most severe forms of human diabetes. It seems not improbable that this physiological action of the glucoside is more closely connected with the sugar portion of the molecule than with the aglykon (non-sugar moiety). Fromm[21] states that the minimal dose of phloridzin required to induce glycosuria in a dog weighing 7 kg. is 1 mg., whereas the threshold value for diabetic action in the case of phloretin is 250 mg.

To these two exceptional properties (lability towards acids; diabetic action) attributable to phloridzin may be added the observation of Dann and Quastel[22] that phloridzin is the only glucoside, of those examined, which exerts a marked retarding effect on the rate of fermentation of glucose by zymin.

The Purification of Phloridzin

The general lack of agreement in the values of the simpler physical constants recorded by various workers for phloridzin may, perhaps, be ascribed to the presence of a small quantity of a compound closely similar to phloridzin—a view which is rendered probable by the fact that phloretin is known to occur in nature combined with sugars other than glucose. Thus glycyphyllin is a condensation product of phloretin with rhamnose, a methylaldopentose.[23] Phloridzin dihydrate is said to melt at 108°C.[24] or 109°C.[25], and to exhibit a specific rotation (D line) of $-49.0°$. The phloridzin used in this work was purified by repeated fractional crystallization from water. The dihydrate thus obtained melted at 113.5 to 114.0°C.; a 1.35 per cent solution in absolute alcohol gave

$$[\alpha]^{20°C.}_{5461Å} = -61.48° \text{ and } [\alpha]^{20°C.}_{5893Å} = -52.40°.$$

Previous Work on the Enzymic Hydrolysis of Phloridzin

Little is known of the behaviour of phloridzin towards enzymes. Although it is not quite clear as to the precise meaning which is to be attached to the term enzymic specificity in the case of the hexosidases,[26] experiments show that derivatives of β-glucose are generally hydrolysed by emulsin; α-glucosides are hydrolysed by maltase. The optical rotation of phloridzin and the prevalence of β-glucosides in nature suggest that phloridzin is a derivative of β-glucose. Euler,[27] in fact, goes so far as to classify it with the β-glucosides, in spite of the statement made by Armstrong[28] that emulsin is without action upon phloridzin. In this connection it is of interest to note that phloroglucinol-β-glucoside, of which phloridzin is regarded as a derivative, is attacked by emulsin.[14] It is fairly certain, however, that phloridzin, although apparently not hydrolysed by emulsin, is attacked by certain other enzymes occurring in the secretions of certain organisms. Charlier,[29]

by artificially circulating defibrinated blood containing phloridzin through the kidneys of various animals, finds that the kidney of the horse (only) contains an enzyme capable of decomposing phloridzin. The juice secreted by the salivary gland and the hepato-pancreas* of the snail hydrolyses salicin, arbutin, coniferin, convolvulin, quercitrin, salonin, saponin, amygdalin, aesculin, helicin, phloridzin, lactose and maltose. From these results of Bierry and Giaza[30] it is not clear whether phloridzin is hydrolysed by emulsin or maltase, since the secretion examined appears to contain both these enzymes. According to Giaza and Gompel[31] the fresh digestive juice pumped from the stomach of the crab is capable of hydrolysing lactose, raffinose, maltose, several β-glucosides, and phloridzin. The phloridzin in this case may have been hydrolyzed by emulsin, maltase, or saccharase.

Experimental Procedure

In attempting to study polarimetrically the action of emulsin, maltase, and saccharase on phloridzin, a difficulty arises out of the low solubility of this glucoside. At 20°C. phloridzin dihydrate is soluble in water only to the extent of 0.189 gm. per 100 cc., this value varying slightly with the addition of salts to the solution. A saturated solution of phloridzin in water at 20°C. exhibits an optical rotation in a 2 dm. tube of $-0.23°$ ($\lambda = 5461$Å); quantitative conversion of this into glucose would correspond to a final rotation of $+0.10°$. It is evident from this that it is not possible to study the kinetics of the enzymic hydrolysis of phloridzin polarimetrically, since the total rotational change theoretically possible is less than a third of a degree. It is possible merely to detect chemical change and, in some cases, to measure its extent after relatively long periods. In order to do this, "blank" readings must be taken with enzyme and buffer solutions at various stages of reaction to ensure the absence of bacterial attack. Experiments have been conducted throughout at 30°C. with solutions of phloridzin previously saturated in buffer solutions at 20°C. Rotations have in all cases been taken in 2 dm. tubes at 20°C., using the green line of the mercury spectrum ($\lambda = 5461$Å). The pH has been controlled by employing the usual Sørensen HCl-sodium-citrate and Na_2HPO_4-KH_2PO_4 buffer solutions.[32] For enzyme preparations extracted without glycerol, the solutions have been kept sterile by the presence of toluol or thymol. The saccharase preparation employed was optically inactive; 0.1 gm. of emulsin preparation in 100 cc. of solution gave a rotation of $+0.02°$; 1 cc. of maltase extract in 100 cc. exhibited a rotation of $-0.02°$, which changed to $+0.02°$ after 40 hours, and remained constant for the remaining 160 hours. In some cases, as

* A duct from the liver unites with a duct from the pancreas before entering the intestine; the secretion referred to is, presumably, obtained from this "joint" duct.

with solutions containing maltase after prolonged maintenance at 30°C., it became necessary, in order to take optical measurements, to remove the enzyme by centrifugal treatment after adsorption on alumina.

The Action of Emulsin on Phloridzin

The emulsin used was extracted with water from the dry product supplied by the British Drug Houses. As the following table (Table II) shows, phloridzin kept in contact with emulsin for 3 days at 30°C. (pH = 4.45) does not undergo chemical change. At the end of this period the enzyme is still active and readily hydrolyses salicin, so that phloridzin cannot be regarded as a catalytic poison towards emulsin. The optimal pH given by Josephson[33] for the β-glucosidase of emulsin is 4.4. This experiment has been repeated at different temperatures (30°C. to 50°C.) and different pH values (4.45 to 6.98) with the same result. There can be no doubt that emulsin does not hydrolyse phloridzin—a conclusion which is difficult to reconcile with the fact that emulsin attacks most of the naturally occurring β-glucosides and the synthetic β-glucosides, including those which are structurally akin to phloridzin, *e.g.*, β-phenyl-glucoside[34] and phloroglucinol-β-glucoside.[14]

TABLE II

Initial rotation of 0.1 gm. emulsin in 100 cc. solution at pH = 4.45	=	+ 0.02°
Initial rotation of 0.189 gm. phloridzin in 100 cc. solution at pH = 4.45	=	− 0.24°
Initial rotation of emulsin-phloridzin mixture	=	− 0.22°
Rotation of mixture after 3 days	=	− 0.24°
Immediate rotation after addition of 1 gm. salicin per 100 cc.	=	− 2.26°
Rotation of mixture 2 days after addition of salicin	=	+ 0.45°

The Action of Maltase on Phloridzin

The maltase used in this work has been prepared from fresh brewery yeast by a method differing slightly from Willstätter and Steibelt's modification[35] of the procedure originated by Croft Hill[36] and Fischer.[37]

200 gm. of fresh, well-pressed brewery yeast are washed several times with 2,500 cc. of distilled water, filtered in a Buchner funnel, pressed on to dry porous tiles and allowed to crumble into hard granular pieces. After a few days of drying in the air, and then in vacuum over H_2SO_4, the granules are powdered, sieved through a copper gauze (200 mesh) and dried in an oven at 35°C. The light yellow powder (32 gm.) can be kept indefinitely in the dry state. To extract maltase from this

dried yeast 2 gm. of the powder are treated with 1.2 cc. of N-ammonia, 9.4 cc. of water and 9.4 cc. of glycerol. The mixture is kept at 30°C., with occasional shaking, for 3 hours, after which it is filtered. The ammonia is added to neutralise the acidity produced during the extraction of the enzyme.[35] The enzyme preparation thus extracted in the presence of glycerol has been found to be much more stable than the water extracts which are usually prepared. 1 cc. of the clear glycerol-water preparation was found to hydrolyse 30 per cent (rotation falls from +5.98° to +4.90°) of a 2 per cent solution of maltose monohydrate at 30°C. and pH of 6.98 in 45 minutes. The enzyme extract is thus seen to be somewhat more active than that prepared by Croft Hill[36] (20 per cent hydrolysis of 2 per cent maltose monohydrate at 30°C. in 40 minutes). The experiments with maltase have been conducted at 30°C. and pH = 6.98. Values published for the optimal pH for this enzyme are: 6.1 to 6.7 (Isaier[38]); 6.0 to 6.8 (Rona and Michaelis[39]); 6.75 to 7.25 (Willstätter and Bamann[40]).

TABLE III

Initial rotation of 1 cc. of maltase extract in 100 cc. of solution at pH = 6.98	= − 0.02°
Initial rotation of phloridzin in buffer solution	= − 0.25°
Initial rotation of maltase-phloridzin mixture	= − 0.27°
Rotation of mixture after 19 hours at 30°C.	= + 0.04°

The results given in Table III demonstrate that phloridzin is attacked by yeast maltase. During the period considered, the rotation of the maltase extract in a buffered solution remained sensibly constant, so that the final rotation due to phloridzin and its products of hydrolysis is +0.06°. This corresponds to about 90 per cent hydrolysis of the glucoside. By using 3 cc. of enzyme extract it is possible to detect the hydrolysis of phloridzin within less than an hour (negative rotation decreases from −0.036° to −0.25° in 45 minutes). Unfortunately, it cannot be definitely concluded from these results that phloridzin is an α-glucoside, because yeast maltase prepared as described above is known to contain, in addition to α-glucosidase, traces of saccharase, which can be completely removed only by adsorption processes.[40] A test with a 2 per cent solution of sucrose buffered at pH = 4.45 showed that the maltase preparation employed contained a small amount of saccharase (rotation falls from an initial value of +3.12° to 2.15° after 21 hours at 30°C.). It is not known, therefore, whether maltase or saccharase is responsible for the hydrolysis of phloridzin.

The Action of Saccharase on Phloridzin

The saccharase preparation employed, which was purchased from the Digestive Ferments Company, was capable of hydrolysing 10 times its own weight of sucrose in a 10 per cent neutral solution of the sugar at 25°C. in 2 hours. The data of Table IV refer to the action of this enzyme on maltose (1.667 per cent monohydrate), sucrose (1.667 per cent) and phloridzin (0.189 per cent dihydrate) at 30°C. and pH = 4.45. The optimal pH for saccharase probably lies between 4.2 (Michaelis and Davidsohn)[41] and 4.5 (Waldschmidt-Leitz[42]). It is clear from the data given in the second and third columns that the enzyme preparation employed is rich in saccharase and is free from maltase. Phloridzin is attacked by saccharase under the conditions

TABLE IV

Solution Contains 5 Cc. of Saccharase Extract Per 100 Cc. Solution at 30°C. and pH = 4.45

Time (hours)	Rotation of solution (in degrees)		
	1.667% maltose	1.667% sucrose	0.189% phloridzin
0.00	+5.30	+2.46	−0.23
0.33	+5.24	−0.10	−0.14
4.17	+5.26	−0.58	−0.08
20.0	+5.30	−0.74	−0.02
70.0	+5.30	—	−0.02

of experiment, the glucoside being hydrolysed to the extent of over 25 per cent in 20 minutes. Since it has not been found convenient to work with saccharase-free maltase as well as with maltase-free saccharase, it cannot be decided which of these enzymes is responsible for the cleavage of phloridzin. It is reasonable, however, to conclude that phloridzin is attacked by saccharase only, and that the behaviour of the glucoside towards the maltase preparation is due to the presence of saccharase in that product. Wiedenhagen[43] has found that the sucrose-splitting enzyme (saccharase) contains small quantities of α-glucase in addition to β-(γ) fructosidase. α-glucase is said to be inactive at pH = 4.7. If this is true, then it must be concluded that phloridzin is hydrolysed by the γ-hexase, whence phloridzin should be regarded as a derivative of a γ-hexose.

The Sugar of Phloridzin

The behaviour of phloridzin towards emulsin, maltase, and saccharase indicates that this substance can hardly be termed a normal glucoside. The sugar produced during hydrolysis should therefore differ in its properties from those of glucose. Experiment shows this to be the case. A 4.646 per cent solution of phloridzin dihydrate, which was completely hydrolysed in 50 cc. of N HCl at 100°C., and from which the precipitated phloretin had been removed, gave a rotation in a 2 dm. tube of +2.00°, which corresponds to a value of +56.42° for $[\alpha]_{5461}^{20°C.}$ in the case of the hexose liberated, reckoned as anhydrous sugar. A crystalline monohydrate of phloridzin-sugar was prepared by hydrolysis of the glucoside by 0.2 N H_2SO_4, followed by neutralisation with $BaCO_3$, filtration, concentration of the syrup under reduced pressure, and crystallization from a mixture of 4 parts of glacial acetic acid to 1 part of water. A 1.204 per cent solution of this sugar in water gave $[\alpha]_{5461}^{20°C.} = +56.65°$, reckoned as anhydrous sugar—a value in agreement with that found by the previous method. It has been found that the ratio of $[\alpha]_{5461}:[\alpha]_{5893}$ for glucose is 1.267, whence $[\alpha]_D$ for phloridzin sugar becomes +44.61°. Hesse,[44] during a careful polarimetric examination of various sugars, found for the sugar of phloridzin $[\alpha]_D = +45.86$, whereas the optical rotatory power of the sugar prepared from honey, grapes, salicin and amygdalin was +51.98°, +52.86°, +52.06° and +54.18°, respectively. The specific rotation for glucose at the concentration considered, *i.e.*, 3 per cent, is +52.58°. The result given in the present investigation is thus seen to be in agreement with the work of Hesse. Unfortunately Fischer,[20] upon whose authority the structure of glucose has been ascribed to phloridzin-sugar, based the identity on the melting point (204°C.) of the osazone, and gave no value for the optical rotation. Roser,[3] who established the sugar as a hexose by measurements of its copper-reducing power, was unable to identify the sugar with glucose. Schiff,[8] by the heat-decomposition of phloridzin, obtained a syrup which he regarded as glucosan. The specific rotation given by Rennie[10] exceeds that of glucose almost as much as Hesse's value falls short of +52.50°. With the possible exception of the physical constants published by Schunk and Marschlewski,[12] it can be concluded that no satisfactory values have yet been found for the melting point of phloridzin-sugar. In

view of the uncertainty which this casts on the true glucosidic nature of phloridzin, it is unfortunate that Johnson and Robertson, after hydrolysing methylated phloridzin, did not examine the methylated hexose thereby produced.

Through the kindness of Professor J. M. Beattie, M.A., M.D., Bacteriologist to the City of Liverpool, it has been possible to compare the effect of various bacilli on sterile solutions of glucose and phloridzin-sugar. In their behaviour towards *Bacillus shiga* (dysentery), *Bacillus paratyphoid B.*, *Bacillus proteus* X 19, and *Bacillus proteus* (Zenker) the two sugars are alike. *Bacillus pestis* (bubonic plague), however, does not ferment phloridzin-sugar within 24 hours, whereas it does ferment glucose. At a later period, phloridzin-sugar is also fermented.

From the data on the kinetics of the hydrolysis of phloridzin by acids, from experiments on the behaviour of this substance towards the sucroclastic enzymes, and from an examination of the parent sugar, it must be concluded that phloridzin can not be regarded as a normal glucoside. The lability of phloridzin towards acids, and its response to the β-(γ) fructosidase of saccharase suggest that we may here be dealing with a derivative of a γ-hexose. The matter is in need of further investigation, which, for non-scientific reasons, can not be undertaken by the present writer.

SUMMARY

1. Considering previously published data on the velocity of hydrolysis of glucosides by acids, it is shown that phloridzin, judged from the standpoint of the velocity coefficient and the critical increment for hydrolysis, resembles the γ-fructosides (sucrose, raffinose and melezitose) more closely than it does the normal glucosides (salicin, arbutin, maltose, etc.).

2. Previous work on the enzymic hydrolysis of phloridzin shows that it is not hydrolysed by emulsin, but that it is hydrolysed by some other enzyme which occurs fairly freely in nature.

3. The difficulty in examining the enzymic hydrolysis of phloridzin lies in its very low solubility. It has been shown, in confirmation of earlier work, that emulsin is definitely without action on phloridzin at various values of pH and of temperature. This result is difficult to reconcile with the β-glucosidic character commonly ascribed to

phloridzin, and with the fact that emulsin hydrolyses (synthetic) phloroglucinol-β-glucoside, of which phlorizin is regarded as a derivative.

4. Phloridzin is hydrolysed by a yeast maltase preparation, known to contain saccharase. Phloridzin is readily attacked by maltase-free saccharase at 30°C. and pH of 4.45. If the α-glucase of the sucrose-splitting enzyme is (as stated) inactive under these conditions, then the enzyme responsible for the hydrolysis of phloridzin is β-(γ) fructosidase.

5. The sugar prepared from phloridzin differs from glucose in its specific rotation and in its action towards *Bacillus pestis*.

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THE OSMOTIC PRESSURE OF CONCENTRATED SOLUTIONS OF GELATIN IN EQUILIBRIUM WITH SOLUTIONS OF MAGNESIUM CHLORIDE

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I

INTRODUCTION

The osmotic pressures of dilute solutions of gelatin in equilibrium with dilute solutions of electrolytes have been discussed in detail by Loeb,[1] from the point of view of Donnan's theory of membrane equilibrium. In the present work, the equilibrium of concentrated solutions of gelatin and of magnesium chloride has been investigated and an attempt has been made to estimate the effects of some of the factors responsible for deviations from the laws for dilute solutions formulated by Loeb.

Theoretically, the effects of the unequal distribution of ions across a membrane should be small, and the osmotic pressure should be proportional to the "corrected" concentration in mols per litre of solvent, if the protein is equilibrated with pure water or a solution of a neutral salt in the region of the isoelectric point. Observations on gelatin dissolved in water,[2] sodium salicylate,[3] molar calcium chloride[4] as well as the observations recorded below agree with the equation $p(V - b) = RT$ rather than the ideal solution law.

In this work, the thermodynamic formulae which have been pub-

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[1] Loeb, J., Proteins and the theory of colloidal behavior, McGraw-Hill Book Co., Inc., New York, 1922.

[2] Kunitz, M., *J. Gen. Physiol.*, 1926–27, **10**, 811.

[3] Horne, E. V., *Biochem. J.*, 1924, **18**, 1107.

[4] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–28, **8**, 317.

lished recently (Adair[5]) have made it possible to test the validity of the hypothesis that the effects of the unequal distribution of ions are relatively small, and an attempt has been made to determine the relationship between the high osmotic pressures of concentrated solutions and the hydration of the protein.

II

Experimental Methods

The manufacture of osmometers with rigid collodion membranes and methods for eliminating errors in osmometric measurements have been described in a previous paper.[6] The membranes used in this work were filled with gelatin-magnesium chloride solutions prepared from the approximately ash free gelatin supplied by the Eastman Kodak Company, and equilibrated for about 3 weeks in a room at approximately 22°C., with solutions containing from 4.0 to 9.0 equivalents of magnesium chloride per litre. In previous investigations (Adair[5,6,7]), buffer mixtures have been used rather than pure solutions of a single salt, but for the purposes of this work, it appears justifiable to simplify the system by the omission of the buffer mixtures because the observations of Horne[3] show that wide variations in hydrogen ion concentration have no measurable effect on the osmotic pressure of gelatin dissolved in concentrated salt solutions. On account of the viscosity of the solutions and other sources of error, the approximation to equilibrium is less exact than that obtained in previous work on haemoglobin,[6,7,8]) but it is unlikely that the pressures recorded in Table I differ by more than 5 or 10 per cent from the thermodynamic equilibrium values, because the manometer readings returned to within a few per cent of their initial values if they were reset at different levels, and the pressures remained constant for 10 days or more in successful experiments.

At the end of the experiment, the density of the solution was measured and 10 cc. of the solution was diluted for the determination of the chloride content by a modification of Volhard's method in which the protein was destroyed by ashing with sodium peroxide. In a number of experiments the potential difference across the membranes was measured using saturated calomel electrodes. The protein concentration C in grams of dry protein per 100 cc. of protein solution has been calculated from Kjeldahl nitrogen determinations, assuming that 1 gm. of protein contains 0.179 gm. of nitrogen.

Provisional estimates of the corrected concentration c in grams of

[5] Adair, G. S., *Proc. Roy. Soc., A*, 1929, **126**, 16.
[6] Adair, G. S., *Proc. Roy. Soc., A*, 1925, **108**, 627.
[7] Adair, G. S., *Proc. Roy. Soc., A*, 1928, **120**, 573.
[8] Adair, G. S., *Proc. Camb. Phil. Soc., Biol., Sci.*, 1923–25, **1**, 75.

dry protein per 100 cc. of solvent have been made by the application of Formula 1.

$$c = \frac{C}{1 - \cdot 01\ \alpha C} \tag{1}$$

α = approximately 0.7 cc. + 0.5 cc. = 1.2 cc., where 0.7 cc. is approximately equal to the volume of 1 gm. of dry gelatin, corrected for the contraction in volume on solution, and 0.5 cc. is a provisional estimate of the hydration (Moran[9]).

A number of different methods for the estimation of hydration are discussed below.

III

The Relationship between the Osmotic Pressure and the Concentration of Gelatin

The observations recorded in Table I and shown graphically in Fig. 1 show that in the case of gelatin equilibrated with 4 normal and 9 normal solutions of magnesium chloride, the osmotic pressure increases more rapidly than the concentration, even if it is corrected for the volume of the protein hydrate by the application of Formula 1. Over the range of pressures greater than 12 mm. but less than 120 mm., the observations can be represented by the simple formula with two empirical constants which has been applied to haemoglobin[7,8]):

$$p = \frac{K_m C}{1 - \cdot 01\ K_b C} = \frac{K_m c}{1 - \cdot 01\ K'_b c}. \tag{2}$$

C = grams protein per 100 cc. solution.
c = grams protein per 100 cc. solvent.

In the case of gelatin at 22°C., $K_m = 6.54$, $K_b = 10.0$ and $K'_b = 8.8$. In the case of haemoglobin at 0°C., K_m is 2.72 and K_b is 2.42. The molecular weight of the protein, or the average size of the particles if the protein is a mixture, can be calculated from the coefficient K_m if the formula applies to dilute solutions, as shown in a previous paper;[7] unfortunately the majority of our experiments with dilute solutions

[9] Moran, T., *Proc. Roy. Soc.*, *A*, 1926, **112**, 30.

of gelatin failed to satisfy the strict criteria for osmotic equilibrium given in a previous paper,[6] and until further data are available it is not advisable to express any opinion concerning the molecular weight of gelatin or its relationship with K_m. Earlier work on the molecular weight of gelatin has been discussed by Jordan Lloyd,[10] and additional evidence has been given by Eggert and Reitstötter,[11] Adair,[8] and Kunitz.[2]

TABLE I

Osmotic Pressures of Gelatin Solutions in Equilibrium with Magnesium Chloride Solutions at 22°C.

[Cl]″ Gram equiv. $MgCl_2$ per litre of outer fluid	[Cl]′ Gram equiv. Cl per litre of gelatin solution	C Grams of dry gelatin per 100 cc. solution	c Grams of dry gelation per 100 cc. solvent (provisional estimates)	p Observed osmotic pressure in mm. of mercury	$\frac{p}{c}$
3.91	3.93	5.23	5.6	54.9	9.8
3.94	3.71	7.20	7.9	116.9	14.8
4.03	3.76	7.28	8.0	123.5	15.4
6.14	5.68	6.95	7.6	124.3	16.4
6.30	5.86	7.18	7.9	130.7	16.6
9.16	9.12	1.10	1.11	5.4	4.9
8.5		2.07	2.1	17.6	8.3
8.5		2.22	2.3	17.8	7.8
8.57	8.18	5.27	5.6	73.0	13.0
8.96	8.53	5.75	6.2	86.2	14.0
9.03	8.56	6.38	6.9	118.9	17.2
9.10	8.50	6.55	7.1	124.2	17.5
8.95	8.55	9.37	10.6	278.1	26.4
8.99	8.45	9.96	11.3	306.1	27.1

The constant K_b is a volume correction term comparable with "b" in van der Waals' equation $p(v-b) = RT$. It is interesting to observe that gelatin resembles haemoglobin in that K_b diminishes at pressures exceeding 120 mm. The data in Table I indicate that $K_b = 8.33$ cc. at a pressure of 278 mm. and 7.92 at 306.1 mm. Prof. Wolfgang

[10] Jordan Lloyd, D., *Biochem. J.*, 1920, **14**, 147.

[11] Eggert, J., and Reitstötter, J., *Z. physik. Chem.*, 1926, **123**, 363.

Ostwald (personal communication) has found a comparable diminution in van der Waals' "*b*" term for a number of solutions, crystalloidal as well as colloidal. Ostwald and Mündler[12] have obtained a formula which is applicable to a wider range of pressures than the simpler equation of van der Waals.

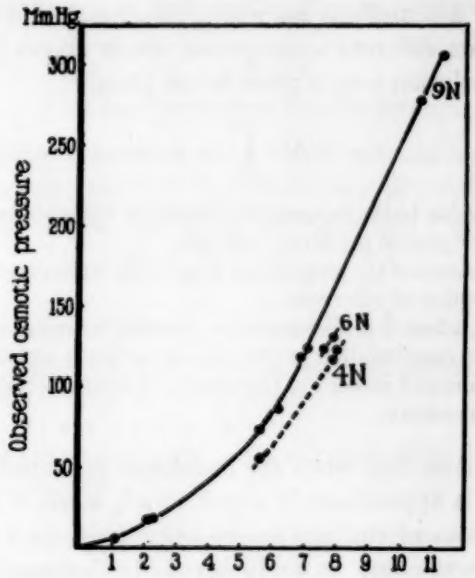

FIG. 1. Concentration of gelatin in grams per 100 cc. of solvent calculated by Formula 1.

IV

The Relationship between the High Osmotic Pressures of Concentrated Solutions of Gelatin and the Membrane Equilibrium of Diffusible Ions

There are at least three different factors which might account for the relatively high pressures observed in concentrated solutions of gelatin, namely, an unequal distribution of diffusible ions, the hydration of the protein, and the effect represented by the term "*b*" in van

[12] Ostwald, Wo., and Mündler, K., *Kolloid Z.*, 1919, **24**, 7.

der Waals' equation, and other deviations from the ideal solution laws.

Sörensen[13] has pointed out that if Donnan's formulae apply to concentrated solutions of electrolytes in equilibrium with approximately isoelectric proteins, the pressure due to the unequal distribution of ions should be small. He drew attention to the uncertainty of some of the assumptions on which this conclusion is based. A formula based on different assumptions which allows for deviations from the ideal solution laws is given below (Adair[5]).

$$p_i = RT \int_0^u m_p n_p \, du \tag{3}$$

p_i = the pressure due to the unequal distribution of diffusible ions.
m_p = gram mols of protein per litre of solvent.
n_p = the mean valence of the protein ions (due to the ionization of the protein or the adsorption of other ions).
u = $E \times F/RT$, where E is the membrane potential measured under conditions where the concentration of the protein is varied and the composition, temperature and pressure of the solution of crystalloids outside the membrane are constant.

It appears probable that when the membrane potential is less than 1 millivolt, $m_p n_p$ is approximately equal to uJ, where J is the sum of the concentrations of the ions in the outer fluid, each multiplied by the square of its valence. In a solution of $MgCl_2$ containing 9 equivalents per litre, $J = 9 + 4.5 \times 2^2 = 27$.

In most of the experiments referred to in Table I, the membrane potentials were so small that they could not be measured with the instruments available, but in the two experiments with approximately 9.3 per cent and 9.9 per cent of gelatin the value of E appeared to be about -0.05 millivolt. By substitution in Formula 1, it was found that the term p_i did not exceed 2 mm. within the range of concentrations referred to in Table I, and therefore the high osmotic pressures cannot be attributed to an unequal adsorption of ions.

This conclusion does not prove that the osmotic pressure of the protein is unaffected by an adsorption of magnesium and chloride ions in equivalent proportions, because the forces of attraction or repul-

[13] Sörensen, S. P. L., *Compt. rend. Laboratoire Carlsberg*, 1917, **12**, 295.

sion between protein molecules might be altered by the adsorption of a neutral salt. Thermodynamical calculations of the effects of adsorption have been made by Polanyi[14] and by Christiansen.[15] The similarity of Kunitz' curves[2] for gelatin dissolved in water and the data for gelatin dissolved in concentrated solutions of magnesium chloride given above seems to indicate that the deviation from the ideal solution laws is mainly due to factors other than adsorption.

V

The Hydration of the Protein

The relationship between the high osmotic pressures of concentrated solutions of proteins and their hydration may be considered from two different points of view, represented by Formulae 4 and 5:

$$\pi (V - v_p) = RT \tag{4}$$

$$\pi (V - \beta v_p) = RT \tag{5}$$

π = the osmotic pressure of a solution of an isoelectric protein.
V = the volume of solution per mol of protein.
v_p = the volume of 1 mol of protein hydrate.
β = a coefficient greater than unity, which may diminish as the protein concentration is increased.

Formula 4 is a restatement of the equation derived by Callendar[16] and other workers on the assumption that hydration affects osmotic pressure by altering the molar fraction of the solvent. The second formula is obtained on the assumption that there may be an analogy between the volume correction term and the "*b*" of van der Waals' " equation for gases. It is known that van der Waals' "*b*" is larger than the volume of the molecules.

The arguments in favour of the simpler Formula 4 have been presented by Kunitz.[2] He finds that if the volume of the protein hydrate ϕ is calculated from the relative viscosity η by his empirical formula $\eta = (1 + 0.5\phi)/(1 - \phi)$,[4] Formula 4 is applicable to aqueous solutions of gelatin at 35°C. According to the viscosity formula, the hydration

[14] Polanyi, M., *Z. physik. Chem.*, 1925, **114**, 387.
[15] Christiansen, J. A., *Compt. rend. Laboratoire Carlsberg*, 1928, **17**, No. 6.
[16] Callendar, H. L., *Proc. Roy. Soc. A*, 1907–08, **80**, 466.

varies from 7 cc. of water per gram of gelatin in a 1 per cent solution to 4.5 cc. of water in a 9.5 per cent solution.

The arguments against the simple Formula 4 are given below:

1. Kunitz' empirical formula for the viscosity of solutions is based on observations on sugar, and it is by no means certain that the effective volume of sugar in solution is the same as its volume in the dry state. Porter[17] and Callendar[16] have come to the conclusion that sugar is hydrated in solution.

2. The effects of the shape of the molecules on the viscosity has not been estimated. If the gelatin molecule consists of long branching chains of amino acids, its effect in increasing the viscosity may be much greater than that which would be caused by spherical molecules of the same volume.

3. The apparent hydration of gelatin calculated by the viscosity formula or by Formula 4 tends to diminish as the protein concentration is increased, although the change in the activity of water caused by the protein is relatively small. The formulae of Lewis and Randall show that an osmotic pressure of 306.1 mm. is correlated with a diminution of 0.038 per cent in the activity of water. If the degree of hydration is determined by the adsorption equation of Freundlich or the formula based on chemical considerations due to Langmuir,[18] the alteration in the degree of hydration should be less than 0.038 per cent, whereas the changes in the apparent hydration calculated from viscosity measurements may be 50 per cent or more.

4. The apparent hydration estimated by the viscosity formula or by the simple osmotic pressure Formula 4 may be from 5 cc. to 10 cc. of water per gram of dry gelatin, whereas the hydration estimated by Moran[9] from the concentration of a gelatin gel in equilibrium with ice at $-20°$C. or lower is about 0.53 cc. per gram of dry protein. Moran's conclusion that the hydration of gelatin is relatively small is in agreement with the chloride distribution measurements recorded in Table I. As a first approximation, the chloride distribution may be correlated with the volume of the isoelectric protein hydrate by an equation of the form stated below.

[17] Porter, A. W., *Trans. Faraday Soc.*, 1917, **13**, 123.

[18] Langmuir, I., *J. Am. Chem. Soc.*, 1916, **38**, 2221.

$$\alpha = \frac{1}{x}\left(1 - \frac{[Cl]'}{[Cl]''}\right) + \frac{b}{[Cl]''} \qquad (6)$$

$\alpha = v_p/M$, where v_p = the volume in cubic centimeters of 1 mol of protein hydrate.

M = grams of dry protein per mol of protein hydrate.

x = grams of dry protein per cubic centimeter of solution.

$[Cl]'$ = gram mols of chloride ion per litre of protein solution.

$[Cl]''$ = gram mols of chloride ion per litre of "outer fluid."

b = gram mols of chloride ions combined with 1000 grams of protein.

If we assume that b is not greater than 1.2 mols, the maximum chloride combining capacity given by Cohn,[19] the value of α given by Formula 6 is from 0.8 to 1.3 cc. The preliminary measurements recorded in Table I are not accurate enough for an exact estimation of α, but it is obvious that they are consistent with the low values obtained by Moran rather than the high values calculated by Kunitz from viscosity measurements.

Further work is necessary before we can arrive at a final decision concerning the hydration of gelatin, but on the whole the evidence now available indicates that the term ϕ in Kunitz' viscosity formula is different from and probably greater than the volume of the protein hydrate.

Since the effects of the unequal distribution of ions and the effects of hydration estimated by the methods which seen most reliable are insufficient to account for the high osmotic pressures of concentrated solutions of gelatin, it seems necessary to assume that there are interionic or inter-molecular forces which become important in concentrated solutions. From the theoretical point of view this conclusion is not improbable because the correction term "b" in van der Waals' equation is larger than the volume of the gas molecules. A similar observation has been made in the case of solutions of haemoglobin, where the term "b" in the osmotic equation, $p(V - b) = RT$, appears to be considerably larger than the volume of the protein hydrate.

Experimental evidence bearing on this problem has been published in a previous paper,[7] and further evidence is given in the following section.

[19] Cohn, E. J., *Physiol. Rev.*, 1925, **5**, 349.

VI

The Relationship between Hydration and the Density of a Protein

If a protein is hydrated, the difference between the density (D') of the protein solution and the density (D'') of the solution of crystalloids

TABLE II

Densities of Haemoglobin Solutions at Approximately 17°C.

Expt. No.	Outer fluid. Composition	Outer fluid. Density	Inner fluid. Density	x	$\frac{D' - D''}{x}$
246	NaCl† 0.15 N	1.005	1.056	0.204	0.250
245	NaCl 0.5 N	1.020	1.065	0.195	0.232
259	NaCl 2.0 N	1.080	1.116	0.197	0.183
243	NaCl 4.0 N	1.152	1.172	0.191	0.107
251	KCl 0.025 N	1.003	1.050	0.190	0.250
250	KCl 0.1 N	1.013	1.060	0.194	0.242
260	KCl 2.0 N	1.019	1.124	0.196	0.168
	Dextrose 1.0 N	1.072	1.107	0.182	0.191
	Dextrose 2.0 N	1.147	1.170	0.187	0.123

† The solutions with NaCl are buffered by 0.025 molar $NaHCO_3$ at pH 8.3. The solutions with KCl are buffered with phosphates at pH 7.9. The dextrose solutions are unbuffered.

TABLE III

Osmotic Pressures of Haemoglobin in Equilibrium with M/10 KCl buffered with 0.0613 molar Na_2HPO_4 + 0.00533 molar KH_2PO_4

Expt. No.	Grams Hb per 100 cc. solution	Litres of solution per gram mol	Pressure p_p in mm.Hg at 0°C.	"b"	α'
300	8.12	823.0	27.8	143	2.1
298	24.00	279.0	140.9	158	2.4
299	34.41	194.0	332.6	211	3.2

"b" = volume correction in litres, calculated by the equation $p_p(V - b) = RT$.

α' (the apparent value of α) $= \frac{b \times 1000}{66{,}800}$

in equilibrium with the protein solution must be affected by the amount of water combined with the protein. In the ideal case of an isoelectric protein which has no effect on the activity coefficients of the crystal-

loids, the relationship can be expressed in the simple form given below.

$$\frac{D' - D''}{x} = 1 + W - \alpha D'' \qquad (7)$$

x = grams of dry protein per cubic centimeter of solution.
W = grams of water of hydration per gram of dry protein.
α = volume in cubic centimeters of protein hydrate per gram of dry protein.

Table II gives a number of observations of the densities of solutions of haemoglobin. The numbers of the experiments have been recorded in order to facilitate reference to the tables given in a previous paper (Adair[7]), which give osmotic pressures and other data not recorded in Table II. The observations in Table II can be represented by Formula 7 with an error not exceeding 0.1, if α is equal to 0.963 cc. and $W = 0.22$ cc. of water per gram of protein. These figures may require correction when we have more data concerning the effect of proteins on the activities of crystalloids, but it is interesting to notice that α calculated from density determinations with different solvents is approximately equal to the value 0.965 calculated from chloride distribution measurements recorded in a previous paper[7] and is much less than the apparent values (α') calculated from osmotic pressure measurements given in Table III by the formula $p(V - v_p) = RT$. In addition to the 3 experiments recorded in Table III, 15 similar experiments are recorded in tables numbered 10 and I in previous papers (Adair[5,7]). The values of α' calculated from the earlier less accurate experiments such as those numbered 200–205 are not very different from the figures recorded in Table III.

SUMMARY

The osmotic pressures and the membrane equilibrium of chloride ions have been determined for solutions of gelatin in equilibrium with solutions of magnesium chloride containing from 4.0 to 9.0 equivalents per litre.

The pressures increase more rapidly than the concentration, an effect represented by a high value of the term "b" in van der Waals' equation $p\,(V - b) = RT$. Calculations made by a thermodynamical formula which makes allowances for deviations from the ideal solution

laws show that the high value of "*b*" is not due to an unequal distribution of diffusible ions.

The theory that the high values of the hydration estimated from viscosity formulae account for the magnitude of "*b*" has been examined and the conclusion has been reached that the term "*b*" for gelatin as well as for haemoglobin is considerably larger than the volume of the protein hydrate.

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